A GENETIC SURVEY OF

TETRACYCLINE RESISTANCE IN GROUP D STREPTOCOCCI

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UNRESTRICTED

A B S T R A C T

This genetic survey of tetracycline resistance determinants in Fife, Scotland, examined 30 isolates of Group D streptococci from both sewage and clinical sources. Similar surveys have been undertaken in the United States on Group B streptococci and oral streptococci but there are no reports of surveys having been carried out in Britain to investigate tetracycline resistance in Group D.

Experiments were undertaken to determine the frequency of transfer and the location of the tetracycline resistance genes. The frequency of transfer of the determinant ranged from 10^{-9} to 10^{-5} per donor, and plasmid DNA was found in 17 of the original donor strains and 4 of the transconjugant strains. The range in plasmid size was 2.7 Md to 36 Md. None of the strains was pheromone or bacteriocin producers. Introduction of pAMβ1. a conjugative plasmid, into the Group D donor strains mobilized the tetracycline resistance genes in 9 of the strains. In an attempt to correlate plasmid content to tetracycline resistance the 10 parental strains containing a single plasmid were treated with novobiocin in an effort to cure them of their tetracycline resistance. None of the strains lost their tetracycline resistance phenotype and their plasmid profiles before and after novobiocin treatment remained the same in all the strains. Hybridization of chromosomal DNA to two cloned streptococci tetracycline resistance genes, tet M a non-plasmid determinant, and tet L a determinant found on small non-conjugative plasmids, failed to reveal any regions of homology under the stringency

conditions used.

The results of this survey do not reveal the precise location and nature of the tetracycline resistance determinants present in the strains investigated.However,analysis of the results enabled suggestions to be made as to the location of the determinants in a number of strains.The location could be further investigated using techniques such as, transformation of the plasmids into Streptococcus sanguis Challis strain, and restriction enzyme analysis. Cloning of the tetracycline resistance determinants found in these strains would also provide information on the nature of these determinants as well as their location.

A C K N O W L E D G E M E N T S

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D E D I C A T I O N

I dedicate this thesis to my parents without whose financial and moral support the work would not have been undertaken.

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, University of St. Andrews; the Department of Microbiology, University of Manchester and the Leukaemia Research Fund Centre, Institute of Cancer Research, London.

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1. INTRODUCTION

1. INTRODUCTION

1.1. A brief synopsis of bacterial plasmids

The discovery of the sex factor in Escherichia coli K-12 in 1952 (Lederberg, J. et al., 1952) opened up a whole new field of bacterial genetics, namely that of extrachromosomal hereditary elements. Bacterial nomenclature of recent years has designated the name plasmid to such elements. Plasmids are autonomously replicating stably inherited molecules of extrachromosomal deoxyribonucleic acid (DNA), not covalently linked to the chromosome, with a molecular weight range of 1 megadalton (Md) to 200 Md. The majority of bacterial plasmids exist as closed circular DNA in vivo, indicating that the double stranded supercoiled DNA helix is intact and has no breaks in either of the two polynucleotide chains.

Plasmid genes code for a diversity of functions which often enable their host bacterium to compete more successfully for survival under hostile conditions, e.g. resistance to antibiotics and toxin production. (For general review see Freifelder, D. 1983; Day, M.J. 1982; Hardy, K., 1981 and Broda, P., 1979a).

1.1.1 Replication

A bacterial strain can harbour more than one type of plasmid which can be inherited stably from generation to generation. Incompatability can be defined as "the inability of two distinct plasmids to be stably co-inherited

in a single clone of dividing bacteria in the absence of continued selection pressure for both plasmid types" (Timmis, K.N. 1979). The elimination of a plasmid from a bacterial strain is known as curing and can be achieved by the use of a number of agents such as acridine orange, which interferes with the replication of the F plasmid and several other plasmids. Plasmids found in members of the enterobacteria can be divided into two groups: large plasmids, which are usually conjugative, with molecular weights of greater than 40 Md and present at only one or two copies per cell, and non-conjugative plasmids which have molecular weights of less than 10 Md and exist in the bacterial cell at about fifteen copies (Williams, P.A. 1978).

Two different types of time-dependant plasmid DNA replication are known to occur in bacteria (Williams, P.A. 1978). Transfer replication takes place during conjugation, when the single strand remaining in the donor cell and the complementary strand transferred to the recipient are replicated to form a double stranded DNA plasmid molecule in both cells. This type of replication has been well studied in the small multicopy non-conjugative ColE1-K30 plasmid from Escherichia coli (Bedbrook, J.R. et al 1979; Itoh, T. & Tomizawa, J. 1979; Staudenbauer, W.L. 1978; Hardy, K. 1981). This plasmid has the capacity to code for eight proteins, including the antibacterial colicin E1 protein. The plasmid has a molecular weight of 4.6 Md and it relies entirely on host enzymes in order to replicate itself. There are three

distinct stages of plasmid replication; initiation, elongation and termination. Replication is initiated at a fixed locus, the origin, and proceeds unidirectionally from that locus. The enzyme DNA-dependent ribonucleic acid (RNA) polymerase, which is synthesized by the host cell, catalyses transcription of a number of bases close to the origin, and this produces an RNA primer for the synthesis of DNA. The synthesis of DNA starts at the 3'OH end of the primer strand and proceeds in a 5' to 3' direction. The DNA synthesis is catalysed by the enzyme DNA polymerase I. After the synthesis of approximately 500 nucleo- tides of the primer strand, discontinuous replication of the complementary strand is initiated. Okazaki fragments of 1,000 bases are produced from small RNA primers which are increased in length by DNA polymerase III holoenzyme. These RNA primers are then substituted by DNA. The enzyme responsible for this stage is probably DNA polymerase I, and the fragments of DNA are linked together by DNA ligase Hardy, K. 1981).

In general most plasmids seem to have a unique locus of replication. Elongation from this locus may be unidirectional, e.g. ColE1 and RK2, or bidirectional, e.g. mini-F. Some plasmids have two sites at which initiation occurs, e.g. R6K and NR1. After initiation the replication fork continues to move until terminated, by reaching a stretch of DNA that prevents subsequent elongation of the strand, or returning to the origin of replication, or by encountering a replication fork moving in the opposite direction (Thomas, C.M. & Helsinki, D.R. 1979).

A high proportion of the large conjugative plasmids of the enterobacteria are able to replicate in the presence of low levels of DNA polymerase I, unlike the small non-conjugative plasmid which require higher levels of the enzyme. These large conjugative plasmids have the capacity to code for one or more of the proteins required for the initiation of their replication. Experiments have shown that in Escherichia coli most of the enzymes required for chromosomal replication are necessary for conjugative plasmid replication, with the exception of the DNA A protein which is needed only in the case of chromosomal replication (Hardy, K. 1981; Thomas, C.M. & Helsinki,D.R. 1979).

Vegetative replication occurs during the normal cell cycle and it ensures that on cell division each of the daughter cells inherits a plasmid. It starts at a different origin to the transfer origin and involves different plasmid genes. The control of vegetative replication is governed by the size of the plasmid (Williams, P.A. 1978). As mentioned previously, plasmids with a molecular weight greater than 40 Md have a low plasmid copy number per chromosome and are under stringent replication control. Plasmids with molecular weights less than 40 Md have a higher copy number and are said to be under relaxed control. Plasmid copy number is also dependent upon a number of factors besides size, such as the state of the cells when they were harvested, for it has been found that cells harvested in the exponential phase of growth, have a lower copy number than

those harvested in the stationary phase. It is currently believed that the regulation of plasmid copy number is due to the binding of a plasmid-encoded repressor to a plasmid operator which results in the inhibition of plasmid replication (Freifelder, D. 1983). It also appears that relaxed control is due to a simpler control mechanism or even a lack of the replication control genes, that are responsible for stringent control of plasmids, which keep vegetative replication and chromosomal replication in phase (Fuke & Inselburg, 1972; Thomas, C.M. and Helsinki,D.R. 1979; Broda, P. 1979a).

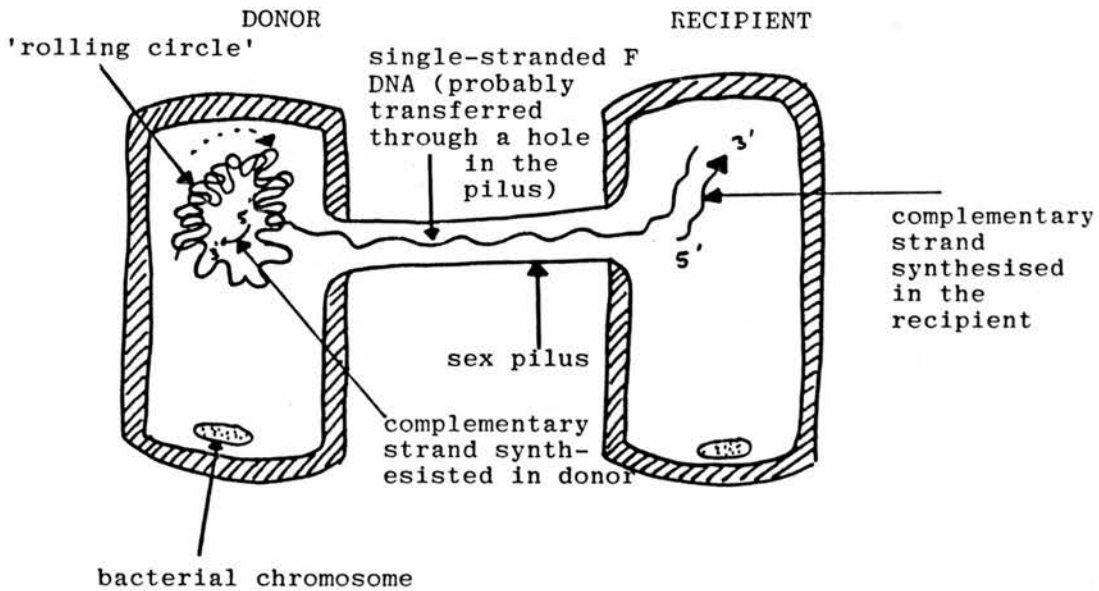
Experimental evidence indicates that plasmid DNA is attached to the bacterial cell membrane during some phase of the cell cycle and that the same proteins may be involved in both attachment to the membrane site and in the initiation of the replication. It is also thought that the attachment of the plasmid to the cytoplasmic membrane, which segregates into the daughter cells, might be responsible for ensuring that each daughter cell receives a copy of the plasmid(Jacob, F. et al 1963; Broda, P. 1979a). The membrane attachment theory may also be the key to plasmid incompatability, for plasmids from the same incompatability group may compete for the same binding sites in the membrane in order to replicate or segregate (Hardy, K. 1981). The enterobacteria can be divided into twenty-five incompatabilty groups, Staphylococcus into five groups and Pseudomonas into at least eleven groups. Plasmids belonging to the same group appear to be related to each other in some

way however, (Datta, N. 1979; Timmis, K.N. 1979; Broda, P. 1979; Day, M.J. 1982; Freifelder, D. 1983). at present the molecular mechanism that gives rise to incompatibility has not been elucidated.

1.1.2. Conjugation

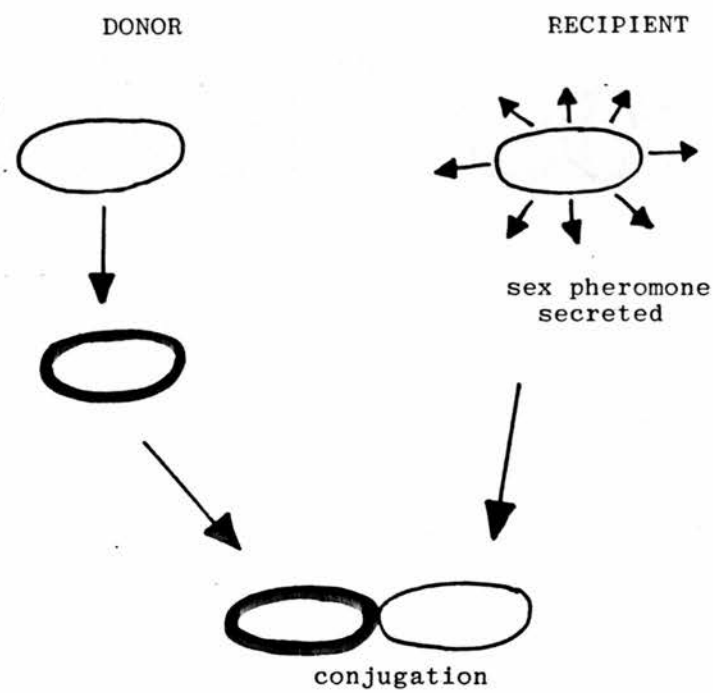
Many plasmids are conjugative, that is they possess a group of genes which enable the plasmid to transfer DNA from a donor bacterium to a recipient bacterium by cell-to-cell contact (Figure 1) (Broda, P. 1979b; Hardy, K. 1981; Willetts, N. 1981). All known conjugation systems are coded for by plasmids. Plasmids can also be transferred via a bacteriophage by a process known as transduction. A third method by which plasmid DNA can be taken up, from solution, into bacterium, is by tansformation. This latter method usually requires special conditions such as pre-treatment of

FIGURE 1



Transfer of plasmid DNA during conjugation (From Hardy, K., 1981, "Bacterial Plasmids".)

FIGURE 2



Expression of sex pheromone by a recipient Streptococcus faecalis strain and response by a donor containing a pheromone-sensitive conjugative plasmid. (From Clewell, D. B. 1981 Microbiological Reviews, 44, pp. 409-436)

the recipient cells with calcium chloride. In nature conjugation appears to be the most prevalent of the three systems for spreading plasmids throughout bacterial populations.

In the gram-negative enterobacteria the sex factor or F plasmid of Escherichia coli K-12 has been the subject of much research (Glass, R.E. 1982; Broda, P. 1979b). It has a molecular weight of 63 Md and has a cluster of 21 genes called the transfer (tra) operon which code for proteins necessary to transfer the F plasmid by conjugation. Some plasmids possess a similar tra region to that of the F plasmid, and these plasmids are known as F-like plasmids (For review see Willetts, N. & Skurray, R., 1980). Heteroduplex analysis shows homology between the conjugation genes of the F plasmid and the F-like plasmids. F-like plasmids produce repressors which exert a negative regulatory control over the transcription of their tra operon, but this repression is not seen in the F plasmid. The process of conjugation in Escherichia coli can be divided into two stages, the first of which is the formation of a mating pair, which does not occur in the processes of transduction and transformation. The sex pili, which are tube-like projections on the surface of the bacterial cell wall, and which are coded for by the plasmid are essential at this stage, because no mating pair can be formed if the pili is removed, e.g. by shearing (Tomoeda, M. et al 1975). The second stage is that of DNA transfer and metabolism. In this stage the heavy strand of the donor plasmid DNA is severed, usually at the origin of transfer, and it is

transferred, 5' end first, into the recipient. It seems likely that plasmid replication in the recipient occurs via the "rolling circle" mechanism and complementary strands are synthesized in both recipient and donor to form an intact duplex (Hardy, K. 1981).

Copies of chromosomal genes can also be transferred between bacteria using conjugative plasmids as vectors, but the frequency of this type of transfer is usually much lower than that of the transfer of the plasmids themselves. The transfer of an entire chromosome by a plasmid hardly ever occurs, but it is obvious that this type of transfer plays an important part in bacterial evolution (Glass, R.E. 1982; Broda, P. 1979a; Broda, P. 1979b).

It is quite common for conjugative plasmids to mobilise and subsequently transfer, non-conjugative plasmids at frequencies approaching those of the conjugative plasmid alone. This mobilisation is independant of the bacterial rec A+ gene product.

Usually mobilisation relies on the nicking, by relaxation proteins, of a target site on the heavy strand of closed circular DNA: this causes relaxation of the duplex and one of the relaxation proteins guides the 5' end of the plasmid into the recipient (Broda, P. 1979a).

Alternatively, homology such as a transposon common to two plasmids, may give rise to an efficient mobilisation.

The presence of conjugative plasmids has been confirmed in most species of gram-negative bacteria. Only four genera of gram-positive bacteria have been shown to contain conjugative plasmids, Streptococcus (For review see Clewell, D.B., 1981), Streptomyces (Troost, T.R. et al., 1979), Clostridia (Blaschek, H.P. & Solberg M., 1981; 1980; Pan-Hou, H.S.K. et al., 1980; Rood, J.I. et al., 1978; Brefort, G. et al., 1977),Bacillus (Battisti,L.et al.1985).

Transfer of plasmids in the genus Staphylococcus is achieved by transduction (Lindburg,M.& Novick,R.P.,1973;Broda,P.1979a).However, Lacy has demonstrated a second mechanism for plasmid transfer which involves cell-cell contact and occurs in the apparent absence of phage DNA.This type of plasmid transfer has been termed 'phage mediated conjugation'(Lacy,R.W.1980),but phage involvement has not been demonstrated conclusively.Drug resistance markers such as gentamicin resistance, which is encoded by the transposon Tn3851 can be transferred between members of the species Staphylococcus aureus and may involve conjugative plasmids and not phages(Townsend,D.E.et al.,1984).The frequency of transfer of conjugative plasmids from Streptococcus faecalis is as high as the transfer frequency of the F plasmid of Escherichia coli($>10^{-1}$ per donor)(Hardy,K.1981). The conjugative plasmids of Streptococcus faecalis code for a sex pheromone, which induces aggregation of donor and recipient cells during mating (Dunny, G.M. et al., 1978) (Figure 2). The pheromone, which is a small peptide with a molecular weight

of less than 1,000 (Clewell, D. et al., 1979), is excreted by the recipient, and the donor carrying a conjugative plasmid specific to the pheromone is attracted to the recipient, and the aggregation occurs resulting in the transfer of plasmid DNA. Once the plasmid has been acquired, the recipient shuts off the production of the pheromone specific to the acquired plasmid. Therefore every conjugative plasmid appears to have a specific pheromone complementary to it. This type of mating process has not been seen in any other species of Streptococcus. The mechanisms involved in conjugation in both Clostridia and Streptomyces have not yet been identified, but they are probably different from the mechanisms involving sex pili, like those found in the gram-negative enterobacteria(Hardy,K.1981).

1.1.3. Types of bacterial plasmids

(a) Virulence plasmids

Pathogenic micro-organisms of the Genera Shigella, Salmonella and the species Vibrio cholerae, are well known for their great contribution to enteric disease, but it should be noted that a number of diarrhoeal infections are caused by gut commensals such as Escherichia coli(Nalin,D.R. et al.,1975;Ryder,R.W.et al.,1976;Williams,P.A., 1978).Infections caused by Escherichia coli are responsible for many deaths amongst neonates and young infants in third world countries. There is also a high fatility from such infections amongst cattle and pigs. It has been shown that many Escherichia coli strains, isolated from such diarrhoeal

infections, carry plasmids which are not present in non-pathogenic strains. Enterotoxigenic strains of Escherichia coli produce Ent Plasmids, which code for either a heat-stable toxin (ST), or for a heat-labile toxin (LT) and ST toxin together(Sack,R.B.,1975;So,M.et al.,1975;So,M.et al.,1976;Smith H.W. and Gyles,C.L.,1970). In the latter case the production of the two toxins seems to be coded for by a single plasmid and no segregation of the two toxin types occurs during conjugation. Both elements of the double toxin, Ent (ST + LT) plasmid from both human and animal sources show a high percentage of DNA homology and have similar molecular weights, ranging from 55 to 60 Md. The Ent (ST) plasmids appear to be more heterogeneous, showing little homology with the Ent (ST + LT) plasmids. The molecular weight range of these plasmids is 20 to 80 Md. Stable toxin is a peptide with a molecular weight of 5,000 and it is coded for by a transposon. The labile toxin component consists of two subunits with molecular weights of 25,000 and 11,500 and this toxin shows a similarity to that of Vibrio cholerae(van Heyningen,S.,1977;Finkelstein,R.A.& Boesman-Finkelstein,M.,1978).

In order to exhibit their maximum capacity for virulence, these enterotoxigenic strains of Escherichia coli require a means by which they can sucessfully populate the intestine. This is achieved by the production of protein pili which enable the pathogenic strains to adhere to the intestinal wall. These pili are often specified by plasmids

different from those which code for the toxins. These pili are produced by K plasmids which code for the production of antigens on the cell surface of Escherichia coli(Smith,H.W.& Linggood,M.,1971;Orskov,I. et al.,1977).

Plasmids have also been implicated in toxin production in Bacillus thurgiensis (Marti, P.A.W. & Dean, D.H., 1979), Clostridium novyi (Schallehn, G. & Kramer, J., 1981), Clostridium perfringens (Blaschek, H.P. & Solberg, M.. 1981; Rood, J.I. et al., 1978; Brefort, G. et al., 1977) and Staphylococcus aureus.

Crown gall disease, a cancer of dicotyledenous plants, is caused by the Ti plasmid of the bacterium Agrobacter tumifaciens (Tooze, J., 1977). A tumour is formed in the plant cells by a fragment of the plasmid. This fragment, T-DNA, is stably maintained in the plant cells which develop tumours. The plasmid also causes the tumour cells to produce opines, derivatives of arginine, which can be used by the bacterium as a source of nitrogen and carbon.

(b) Bacteriocins

" Bacteriocins are antibacterial proteins produced by bacteria"(Hardy,K.,1981). Those bacteriocins which act only on the family of Enterobacteriaceae are known as colicins. The colicins can be divided into two groups(Broda,P.,1979a). Group I Col plasmids are small and non-conjugative, with a molecular weight of approximately 5 Md and a copy number of 10 to 30 plasmids per chromosome, e.g. ColEl-K30. Group II

Col plasmids are large and conjugative and have a molecular weight range of 60 to 100 Md and a copy number of 1 to 3 plasmids per chromosome, e.g. ColV. Colicin plasmids make the host bacterium immune to the colicins encoded by the plasmid present. The site of action of the colicin varies with the colicin type, e.g. ColE1-K30 acts on the cell membrane, whereas ColE3-CA38 acts on the ribosomal RNA of the sensitive bacterium.

Plasmids coding for bacteriocins are ubiquitous and have been demonstrated in a number of bacteria such as Bacillus (Berhard, K., et al., 1978; Rostas, K., et al., 1980). Streptococcus faecalis (Clewell, D. et al., 1979; Oliver, D. et al., 1977), Clostridium perfringens (Li, A.W. et al., 1980; Rood J.I. et al., 1978) and Serratia marcescens (Eichenlaub, R. & Winkler, U., 1974).

(c) Degradative plasmids

Degradative plasmids allow members of the genus Pseudomonas to grow on a diversity of chemicals such as toluene, xylene, and camphor (for reviews see Wheelis, M.L., 1975; Chakrabarty, A.M., 1976; Farrell, R. & Chakrabarty,A.M., 1979). The enzymes encoded on the plasmids convert these chemicals into substrates which can then enter the metabolic pathways of the bacterium. Some degradative plasmids can be transferred via conjugation from strains of the Pseudomonas onto strains of Escherichia coli.

Salmonella spp. are usually unable to utilize lactose

but a few strains of Salmonella typhi have plasmids which enable them to ferment lactose. Plasmids coding for lactose-utilizing enzymes have also been found in strains of Serratia, Proteus and Streptococcus lactis (Kuhl, S.A. et al., 1980).

A numbers of strains of Rhizobium (Nuti, M.P. et al., 1979) contain plasmids encoding nif genes which endow the Rhizobium with the ability to nodulate and fix nitrogen in the roots of leguminius plants. This has obvious effects on agriculture, especially on the use of fertilizers.

Another property bestowed on some bacterial genera by plasmids is resistance to metals. Cases of mercury resistance have been repoted on strains of Pseudomonas (Stanisich, V.A. et al., 1977) and Staphylococcus aureus (Novick, R. & Roth, C., 1968; Weiss, A.A. et al., 1977). Plasmids conferring resistance in nickel, cobalt, mercury and arsenate have been demonstrated in both Escherichia coli (Wang, P.Y. et al., 1978) and Salmonella (Schottel, J. et al., 1974).

(d) Cryptic plasmids

Analysis of the plasmid content of many bacterial genera have revealed the presence of circular DNA of low molecular weight, often less than 10 Md. These plasmids do not seem to have a specific phenotypic trait or biological role, but this does not mean that they do not have a function, hence the name cryptic plasmids(Day,M.J.,1982).

(e) Resistance plasmids

Plasmids which confer resistance to antibiotics are of great importance both in human and vetinary medicine, because of the problems they pose in the therapeutic treatment of bacterial infections. In general, antibiotic resistant bacteria isolated in the wild do not contain chromosomal mutations to antibiotics, but carry their resistance genes on plasmids(Hardy,K.1981). Plasmid and chromosomal resistance to the same antibiotics is achieved by different mechanisms. Many resistance plasmids code for a number of antibiotic resistances, that is, they are multiple resistant, and these resistances can be transferred from one bacterium to another via conjugation. Thus, antibiotic resistance can be efficiently disseminated between members of different genera.

The discovery of resistance plasmids was made in Japan in 1957 following the succession of Shigella epidemics. Antibiotic therapy for the treatment of Shigella was started in 1946 and by 1964 fifty per cent of Shigellae were multiple resistant to the four antibiotics used to treat the pathogen. Strains of Escherichia coli isolated from patients carrying strains of multiple resistant Shigella often shared a similar antibiotic resistance pattern and this led the Japanese microbiologists (Ochiai, K. et al., 1959; Akiba, T. et al., 1960) to implicate as resistance transfer vectors, since multiple resistant Escherichia coli strains could transfer their resistances to antibiotic-

sensitive *Shigella spp.*, by cojugation both under laboratory conditions and in patients. The discovery of resistance plasmids in the enterobacteria by the Japanese soon led to many cases involving plasmid mediated antibiotic resistance being reported from all over the world. In general the resistance plasmids of gram-negative bacteria are conjugative, whereas the majority of those found in gram-positive bacteria tend to be non-conjugative(Hardy,K.1981).

The enterobacteria often harbour resistance plasmids that are F-like, that is, they show similarities, both in DNA sequence and in their *tra* operon genes, to the F plasmid(Broda,P.1979a). F-like resistance plasmids can generally be divided into two regions (a) the *r-determinant*, an area of genes grouped closely together that code for antibiotic resistance, flanked on either side by a copy of the IS1 insertion sequences, and (b) the *resistance transfer factor* (RTF) which comprises the genes necessary for plasmid replication and conjugation makes up the rest of the plasmid(Hardy,K.1981).

Resistance genes are often carried on plasmids by discrete genetic entities known as transposons. These are elements, capable of *rec* A^+ independant translocation, which can insert into a DNA sequence with which they show no sequence homology. They are literally "jumping genes" and range in size from 4 to 21 kilobases(Glass,R.E.1982).Transposons can be divided into

three classes(Freifelder,D.1983):-

a)IS elements and composite transposons

IS elements are sequences of DNA,0.75 to 1.5kb long,that encode at least one protein which is essential for transposition.These sequences sometimes contain a second protein which might play a regulatory role.A number of transposons encoding antibiotic resistance,which are themselves part of other transposons,comprise a transposable genetic element flanked by an insertion element and are known as composite transposons.The flanking insertion elements may be either identical or nearly identical to each other and they may be in either an inverted or direct repeat configuration.Examples of this type of transposon are Tn5,which encodes resistance to kanamycin(Rothstein,S.J.et al.,1981)and Tn10 which encodes resistance to tetracycline(Foster,T.J.,1983).

b)The Tn3 family of transposons

The members of this family of transposons are quite large,approximately 5kb.Each transposon encodes three genes(Heffron,F.et al.,1979).The genes are a -lactamase,which confers ampicillin resistance,and two genes required for transposition.Members of the Tn3 family contain 38 base pair inverted repeats but none of the members are flanked by insertion elements.

c)Transposable phages The phages Mu and D108 integrate their DNA into the chromosome of the host cell early in infection at a number of sites in the chromosome.This produces a characteristic transposon sequence - phage DNA flanked by duplicated target sequences.

Transposons have been found in both gram-positive and gram-negative bacteria (Table 1). Insertion sequences are small fragments of DNA of 700 to 1500 base pairs, often containing inverted terminal repeats. These elements, like transposons, can also translocate themselves and insert into regions with which they share sequence homology. Apart from their transposable ability, research has not revealed the precise function of insertion elements.

There are a number of ways in which plasmid mediated resistance can be effected, (for review, see Davies, J. & Smith D.I., 1978; Forster, T.J., 1983).

TABLE 1

TYPES OF TRANSPOSONS

Transposon	Size in Kilobases	Phenotype	Repeated Sequences
Tn 1	4.8	Ap^r	Short, inverted 38 base pairs
Tn 2	4.8	Ap^r	Short, inverted 38 base pairs
Tn 3	4.8	Ap^r	Short, inverted 38 base pairs
Tn 4	20.5	$Ap^r Sm^r Su^r$	Short, inverted 38 base pairs
Tn 402	7.6	Tp^r	?
Tn 5	5.2	Km^r	Long, inverted 1,400 base pairs
Tn 501	7.9	Hg^r	Short, inverted
Tn 551	5.2	Em^r	Short, inverted
Tn 554	6.0	$Em^r Sp^r$	
Tn 6	4.1	Km^r	Short
Tn 7	12.9	$Tp^r Sm^r$	Short, inverted
Tn 9	2.5	Cm^r	Long, IS1
Tn 903	3.1	Km^r	700 base pairs Long 1050 base pairs
Tn 917	5.1	Em^r	Short, inverted 280 base pairs
Tn 951	16.6	Lac	Short, inverted 100 base pairs
Tn 10	9.3	Tc^r	Long, inverted 1,400 base pairs
Tn 1681	2.9	Ent	Long, inverted Is1, inverted
Tn 1696	13.7	Gm^r, Sm^r, Su^r Cm^rm Hg^r	Short, 140 base pairs
Tn 1699	9.0	Ap^r Km^r Cm^r	Short 50 base pairs

Abbreviations

r - Resistance Ap - Ampicillin Sm - Streptomycin
Su - Sulphonamide Tp - Trimethoprim Km - Kanamycin
Hg - Mercuric salts Em - Erythromycin Sp - Spectinomycin
Cm - Chloramphenicol Tc - Tetracycline Lac - Lactose fermentation
Ent - Enterotoxin production

Compiled from Klecker, N. (1981). Ann. Rev. Genet. 15: 341-404, and Hardy, K. (1981). Bacterial Plasmids, p. 53. Nelson.

1. Enzymic substitution of an enzyme produced by the host bacterium, which is the normal site of antibiotic action,

e.g. the sulphonamides and trimethoprim.

Sulphonamides resistance plasmids in gram-negative bacteria encode the genes which constitutively synthesise a dihydropteroate synthetase, which is unaffected by sulphonamides, and can therefore provide the bacterial cell with dihydrofolate, without competition from the bacteriostatic sulphonamides. In the case of the antibiotic trimethoprim, the resistance plasmids of gram-negative bacteria code for a dihydrofolate reductase resistant to trimethoprim. The synergistic action of trimethoprim and the sulphonamides on the folate co-enzyme pathway means that these two drugs are often prescribed together.

2. Modification of the antibiotic target site,

e.g. erythromycin and lincomycin.

Plasmids isolated from strains of streptococci and staphylococci that are resistant to erythromycin and lincomycin encode methylating enzymes which modify the 23S RNA molecules of bacterial ribosomes and thus prevent the binding of these bacteriocidal antibiotics to their target site.

3. Changing the antibiotic so that it becomes inactive,

e.g. chloramphenicol,penicillins and aminoglycosides.

An example of antibiotic resistance due to this

mechanism is the production, by a plasmid of the enzyme chloramphenicol acetyl-transferase, which inactivates chloramphenicol, thus preventing the bacteriostatic action of the drug, which in the absence of the enzyme, binds to the bacterial ribosomes.

This mechanism is also exhibited in the penicillins where plasmids encode the β-lactamase enzymes, which hydrolyse the β-lactam ring of the drug and therefore prevent the penicillins from killing the bacteria by inhibiting cell wall synthesis.

Aminoglycosides such as streptomycin and gentimicin are bacteriocidal drugs which affect the translation step in protein synthesis,since these antibiotics bind to the ribosomes.Resistance to aminoglycosides is caused by mutations resulting either in reduced binding of the drug to the ribosome or impaired transport across the cytoplasmic membrane.

4. Decreased accumulation involving efflux mechanisms.
   e.g. tetracycline.

The first tetracycline, chlortetracycline, was used clinically in 1948 (for review see, Levy, S.B., 1981). It was isolated by Duggar from Streptomyces aurifaciens and the tetracycline family has continued to remain effective against a wide variety of pathogens, although over the past few years the incidence of both plasmid mediated and chromosomal resistance to the tetracyclines has increased.

What is the mode of action of tetracycline on the bacterial cell? The target site for this bacteriostatic antibiotic is the mRNA of the 30 S ribosome, where it prevents binding of the aminoacyl t-RNA to the A site of the ribosome and this results in the inhibition of protein sythesis. Tetracycline chelates a number of cations; in fact the binding of tetracycline to the ribosomes requires the presence of cations, in particular magnesium, but the precise role of these metal ions in the antibacterial activity of tetracycline is not known.

Concentrations of the drug exceeding the therapeutic level cause permeability changes due to membrane damage, accompanied by a failure of the DNA to replicate itself. Lipid soluble members of the tetracycline family, e.g. minocycline, seem to affect the same target site as tetracycline, although these antibiotics are generally bactericidal when given at a therapeutic dosage.

Studies of tetracycline transport in sensitive cells of Escherichia coli (Arima, A. & Izaki, K., 1963; Franklin, T.J. & Higginson, B., 1970; Levy, S.B. et al., 1977; McMurray, L. & Levy, S.B., 1978; McMurray L.M. et al., 1981) and Streptococcus faecalis (Munske, L.M. et al., 1984; Lindley, E.V. et al., 1984) have shown that tetracycline uptake is an active process involving proton motive force and in addition, in the case of Escherichia coli phosphate bond hydrolysis (Smith, M.C.M. & Chopra, I., 1984). The

drug is accumulated against a concentration gradient, although the initial stages of drug uptake possibly occur via passive diffusion coupled to intracellular binding and this plays a substantial role in inhibiting protein synthesis. Tetracycline is not very lipid soluble although passive diffusion of tetracycline through phospholipid bilayers has been demonstrated (Argast, M. & Beck, C.F., 1984). Experiments endeavouring to show the involvement of porins have not proved to be conclusive, therefore the mode of entry of tetracycline has not been elucidated. Active uptake of tetracycline has also been shown in strains of Staphylococcus (Dockter, M.E. & Magnuson, J.A., 1975), Bacteroides (Fayolle, F. et al., 1980), Bacillus megataterium (Dockter, M.E. et al., 1978) and Rhodopseudomonas (Weckesser, J. & Magnuson, J.A., 1976).

In general bacterial resistance to tetracycline is borne on plasmids. This is not the case though for Proteus mirabilis where the resistance is thought to be carried by a determinant on the chromosome. To date, in gram-negative bacteria, five tetracycline resistance determinants have been identified using DNA:DNA hybridization to specific P^{32} genetic probes, and restriction enzyme analysis (Mendez, B. et al., 1980; Levy, S.B., 1981). These determinants have been classified A to E:-

Class A comprises the RP1-like determinants. RP1 is a plasmid originally discovered in Pseudomonas aeruginosa which carries resistance to tetracycline, ampicillin, and

kanamycin. This class shows a low tetracycline resistance and slight or no resistance to the other members of the tetracycline family.

Class B determinants show homology to the transposon Tn 10, found on the R222 plasmid of Shigella flexineri. The members of this class show resistance to both tetracycline and its analogues.

Class C determinants were characterized by a low resistance to tetracycline and sensitivity to its analogues. This determinant was located on plasmid pSC101, isolated from a strain of Escherichia coli, and it shows homology with several plasmids isolated from Salmonella spp.

Class D is composed of a single plasmid member at present. This determinant is carried by the plasmid RA1, isolated from a tetracycline resistant strain of Aeromonas liquefaciens.

Class E determinants are located on phage lambda.

It seems clear from the DNA:DNA hybridization studies of these determinants that the genetic constitution of the different classes is not the same, yet in a study carried out on Escherichia coli strains (Mendez, B. et al., 1980) containing plasmids from classes A, B, C and D, it was revealed that the mechanism for resistance was the same for each of the classes.

Examination of the types of naturally occurring tetracycline determinants in Escherichia coli strains, using genetic probes (Levy, S.B. 1981) showed that 65% of the strains carry the tet B determinant, the other classes being encountered at a much lower frequency. More than one tetracycline resistance determinant was found to be present in about 4% of the strains examined.

Tetracycline resistance determinants have also been demonstrated in gram-positive bacteria. A tet determinant has been characterized on the Bacillus plasmid pAB124 (Eccles, S.J. and Chopra, I. 1984). Three distinct tet determinants have been identified in the genus Streptococcus (Burdett, V. et al., 1982a,b) (see section 1.2.5.) and two determinants in Streptomyces (Ohnuki, T. et al., 1985). The tet determinants found in Bacillus and Streptococcus do not however appear to be related to those found in gram-negative bacteria has yet to be determined.

A structural protein induced by tetracycline called TET was discovered in Escherichia coli in 1974 (Levy, S.B. & McMurray, L., 1974). This protein which is required for tetracycline resistance, was identified using an R plasmid-in-minicell system and it has a molecular weight of 36,000 daltons. TET is located on the inner membrane of the bacterial cell wall and it is associated with transposon Tn 10, which as mentioned previously is found on plasmid R222. The TET structural region of Tn 10, can be defined by two complementation groups tet A and tet B (Curiale, M.S. and Levy, S.B., 1982),which encode the TET protein. The two complementation groups have been shown to be

coordinately expressed as an operon (Coleman, D.C. et al., 1983) which defines a single open reading frame of 1,202 bases coding for a 43,300 dalton protein (Hillen, W. & Schollmeier, K., 1983).

Recent studies have shown the presence of a single tetracycline inducible RNA of approximately 1,200 bases, which is homologous with the tetracycline resistance structural gene region (Curiale, M.S. et al., 1984). Experiments using deletion mutants have indicated that the tet A and tet B complementation regions form two parts of a single gene which encode two domains of the TET protein (Curiale, M.S. et al., 1984).

A protein resembling TET has also been shown to be present on genetic determinants belonging to Class A and Class C (Levy, S.B. et al., 1971; Tait, R.C. & Boyer, H.W., 1978; Gayda, R.C. et al., 1979; Levy, S.B., 1981). These TET proteins are all inducible and all of them seem to be located on the cell membrane but their sizes appear to differ. A membrane protein has also been isolated from Staphylococcus (Avtalion, R.R. et al., 1971; Wojdani, A. et al., 1976).

A tetracycline inducible protein of 25,000 daltons synthezied by Tn 10 has been described. This protein is the product of a repressor gene (Hillen, W. et al., 1982; Hillen, W. & Unger, B., 1982; Wray, L.V. et al., 1981) thus it appears that the induction of tetracycline resistance is mediated by negative regulation since the repressor protein negatively regulates transcription of the Tn 10 tet A gene, as well as the tet R (repressor) gene. (Wray, L.V. et al., 1981). It is of interest

to note that the genes encoding inducible tetracycline resistance in Tn 1721 appear to be organised in the same way as those of Tn 10 (Altenbuchner J. et al., 1983). A repressor control of TET synthesis has been demonstrated (Yang, H.L. et al., 1976), thus it appears that the induction of tetracycline resistance is mediated by negative regulation. Despite the advances made in the molecular biology of tetracycline resistance it is not known what the precise role of tetracycline inducible proteins is in plasmid mediated tetracycline resistance.

Plasmid encoded resistance in the genus Streptococcus (Levy, S.B., 1981) and in strains of Haemophilus (Levy, S.B., 1981) does not appear to be inducible. However an inducible tetracycline resistance determinant has been demonstrated in a strain of Streptococcus faecalis (Le Blanc, D.J. & Lee, N.L., 1982). Inducible resistance has been demonstrated in Escherichia coli (Levy, S.B. & McMurray, L., 1974; Izaki, K. et al., 1961; Unowsky, J. & Rachmeler, M., 1966; Franklin, T.J., 1967), Proteus (Levy, S.B., 1981), Pseudomonas (Levy, S.B., 1981), Staphylococcus (Sompolinsky, D. et al., 1970) and in some strains of Bacteroides (Fayolle, F. et al., 1980). Previous exposure of the micro-organism to the drug increases the level of antibiotic resistance.

How is resistance to tetracycline achieved? All the tetracycline resistance determinants studied to date, in Escherichia coli exhibit an energy dependent efflux system (McMurray, L. et al., 1980). Experiments have demonstrated that the active efflux system in everted vesicles of Escherichia coli

cells show different biochemical characteristics to those of tetracycline sensitive everted vesicles, which display an active influx system. The saturation kinetics of the resistant cells differ in that they become saturated at lower concentrations of the drug than their sensitive counterparts. Also resistant cells display different pH and magnesium requirements. Thus it appears that the tetracycline resistance determinant responsible for the efflux system is plasmid-borne.

Studies in Escherichia coli have also shown that at least four of the five classes of determinants exhibit different levels of efflux which relate to the different levels of tetracycline resistance.

There is no decreased accumulation of tetracycline, in Eschericha coli with increased tetracycline resistance, despite the apparent increase of tetracycline efflux in tetracycline resistant cells. Yet, the tetracycline does not seem to affect protein synthesis (Levy, S.B. et al., 1977; Levy, S.B. & McMurray, L., 1978; Reynard, A.M. et al., 1971). However, differences in the accumulation of tetracycline between different tetracycline resistance determinants in the genus Streptococcus have been observed (Burdett, V., 1985) (see section 1.2.5). Experiments using the chemical toluene to increase the permeability of the cell towards tetracycline (de Smet, M.J. et al., 1978) indicated that efflux and influx differences were the only factors responsible for tetracycline resistance, and that accumulation of the drug is the crucial difference between a cell being sensitive to tetracycline and one being resistant. There

is also the possibility that the toluene could be interfering with membrane factors responsible for tetracycline resistance. At present there does not appear to be an enzyme responsible for inactivating tetracyline. Studies have demonstrated that tetracycline is not degraded (Levy, S.B. et al., 1977; Sompoinsky, D. et al., 1970; Levy, S.B. & McMurray, L., 1978; DeZeeuw, J.R., 1968).

In conclusion, the incidence of tetracycline resistance amongst micro-organisms is increasing. The fact that the drug is not degraded facilitates the selection of resistant bacteria, as the drug remains in the environment. The emergence of bacterial strains resistant to tetracycline still needs to be elucidated before steps can be taken to prevent the development of further resistant strains.

1.2 Plasmids in the Genus Streptococcus

1.2.1. The Genus Streptococcus

The genus Streptococcus comprises micro-organisms that are gram-positive, non-sporing, facultatively or obligatively anaerobic, chemo-organotrophic cocci or coccoid bacteria. These bacteria are usually found in pairs or in chains. A number of species exhibit haemolysis on blood agar.

Streptococci can be classified on the basis of Lancefield streptococci grouping tests, which identifies specific antigenic carbohydrates (C substances) located on the bacterial cell wall. Important members of the genus includes:-

1. Streptococcus pyogenes (β haemolytic, Lancefield group A):- Erythrogenic strains of this group cause scarlet fever and streptococcal throat.

2. Streptococcus agalactiae (β,α, and non-haemolytic, Lancefield Group B), this is the causative agent of bovine mastitis.

3. Streptococcus faecalis (non-haemolytic, although some haemolytic strains have been isolated, Lancefield Group D), is a commensal found on the lower intestine of man. It is also responsible for causing bacterial endocarditis in humans.

4. Streptococcus lactis and Streptococcus cremoris (Lancefield group N), are found in dairy products,

5. Streptococcus mutans is asscoiated with the production of dental plaque.

6. Streptococcus pneumoniae is the causative agent of pneumonia.

1.2.2. A brief historical review of plasmids in Streptococci

1972 - Plasmid DNA was first demonstrated in a species of Streptococcus faecalis which was resistant to tetracycline and erythromycin by Courvalin and colleagues (Courvalin, P.M. et al., 1972).

1973 - Dunny and co-workers isolated and characterized a small plasmid from a strain of Streptococcus mutans (Dunny, G.N.

et al., 1973).

1974 - Jacob and Hobbs showed that plasmids carrying multiple drug resistance could be transferred via conjugation in Streptococcus faecalis (Jacob A. et al., 1975).

1975 - Jacob and co-workers showed the presence of self-transmissible plasmids encoding for the hemolysin and bacteriocin of Streptococcus faecalis (Jacob A. et al., 1975).

1976 - Chassy developed a method for growing streptococci which inhibited cell wall cross-linking, thus enabling the bacteria to be easily lysed and therefore more susceptible to plasmid analysis (Chassy, B.M., 1976).

- Transfer of plasmids by transduction was demonstrated between strains of group N streptococci (McKay, L.L. et al., 1976).
- Transfer of plasmid DNA between different species was achieved by the process of transformation, using the group H Challis strain of Streptococcus sanguis (Le Blanc, D.J. & Hassell, F.P., 1976).

1977 - Van Embden and co-workers described a conjugation system in Group D streptococci which required cell-to-cell contact achieved by mating the donor and recipient strains of bacteria on a membrane filter. (van Embden, J. et al., 1977)

- Efstathiou and McKay demonstrated plasmid-borne resistance to silver, copper, chromate, arsenite, and aresnate in Streptococcus lactis (Efstathiou, J. & McKay, L.L., 1977).

1979 - Clewell described a second type of conjugation system in streptococci namely the sex pheromone mating system of

Streptococcus faecalis (Clewell, D. et al., 1979).

1980 - An erythromycin-inducible transposon, Tn917, found in Streptococcus faecalis was demonstrated by Tomich and co-workers (Tomich, P. et al., 1980).

1981 - A tetracycline resistance transposon, Tn916, was discovered by Franke and Clewell in a strain of Streptococcus faecalis (Franke, A.E. & Clewell, D.B., 1981).

1982 - Burdett showed three typres of resistance determinants in strains of streptococci resistant to tetracycline (Burdett, V. et al., 1982).

- Two tetracycline resistance determinants form Streptococcus faecalis JH1 were characterized by Le Blanc, D.J. & Lee, L.N., 1982).

1983 - Perkins and Youngman demonstrated that the Streptococcus faecalis plasmid pAMα1 is composed of two separate replicons. One of these replicons has been shown to be closely related to the Bacillus subtilis plasmid pBC16 (Perkins, J.B. and Youngman, P. 1983).

- The conjugative R plasmid, pJH1, from Streptococcus faecalis strain JH1 coding for kanamycin, streptomycin, erythromycin and tetracycline was genetically and physically analysed and was found to be similar to the plasmid pAD2 from Streptococcus faecalis strain DS16 (Benai, M. and Le Blanc, D., 1985).

- Modified forms of Streptococcus faecalis pheromones were demonstrated following the acquisition of plasmid DNA by recipient strains. This modification was shown to "shut off" the production of endogenous pheromone (Ike, Y. et

al., 1983).

1984 - The transposon Tn916 was cloned on a plasmid vector in Escherichia coli in an attempt to facilitiate the gentic analysis of this transposon (Gawron-Burke, C. & Clewell, D.B., 1984).

- The transposon Tn917, which encodes resistance to erythromycin, was used to generate a series of insertional mutations in the Streptococcus faecalis plasmid pAD1 in order to perform a genetic analysis of the pAD1 phermone response. (Ike, Y. & Clewell, D.B., 1984)

1985 - The integration of plasmid DNA from two Staphylococcal plasmids into the chromosome of Streptococcus pneumonine was shown to use most commonly flanking homology involving an apparent double cross-over (Pozzi, G. & Guild, W.R., 1985).

- The Streptococcus faecalis sex pheromone cAM373 was also found to be produced by Staphylococcus aureus and Streptococcus sanguis. (Clewell, D.B. et al., 1985).
- Two new conjugative transposons Tn918 (Clewell, D.B. et al., 1985) and Tn919 (Fitzgerald, G.F. & Clewell, D.B., 1985) conferring tetracycline resistance were described in Streptococcus faecalis strain RC73 and Streptococcus sanguis strain FC1 respectively.

1.2.3. Streptococcal plasmids

Since 1972 plasmids have been demonstrated in a number of strains of streptococci (for review see Clewell, D., 1981a). Their occurrence is quite common. In fact, plasmids are found at the same frequency as in gram-negative enterobacteia. Different strains of streptococci show different plasmid profiles. Table 2

TABLE 2

List of Streptococcal Plasmids

Species	Plasmid	Mol wt (x 10^6)	Related phenotype(s) *	Conjugative	Original host
S.faecalis	pAMα1	6.0	Tc'(amplifiable)	No	DS5
	pAMβ1	17	Em'	Yes	DS5
	pAMγ1	35	Hly-Bac, UV', PR	Yes	DS5
	pAMγ2	35	Bac, PR	Yes	DS5
	pAMγ3	35	PR	Yes	DS5
	pAD1	35	Hly-Bac, UV', PR	Yes	DS16
	pAD2	15	Em', Sm', Km'	No	DS16
	pOB1	46	Hly-Bac, PR	Yes	5952
	pOB2	28	Bac (streptocin 101)	No	5952
	pPD1	35	Bac,UV', PR	Yes	39-5
	pPD2	10	Cryptic	?	39-5
	pPD3	5	Cryptic	?	39-5
	pPD4	3	Cryptic	?	39-5
	pPD5	35	Hly-Bac	Yes	39-5
	pAM 539	26	Sensitivity to streptocin 101	?	ND539
	pAM547	9	Cryptic	?	ND547
	pAM81	16	Em'	Yes	DU81
	plP613	18	Em'	Yes	BM6201
	plP614	65	Tc'	Yes	BM6201
	pJH1	50	Em', Tc', Km', Nm'	Yes	JH1
	pJH2	38	Hly-Bac, PR	Yes	JH1
	pJH3	38	Hly-Bac	Yes	JH3
	plP800	70	Km', Gm', Cm'	Yes	BM4100
	plP801	53	Em', Hly-Bac	Yes	BM4100
	plP802	49	Cryptic	Yes	BM4100
	plP803	2	Tc'(?)	No	BM4100
	pJH4	26	Em', Km', Sm'	Yes	JH7 (S. faecium)
	pJH5	76	Em', Tc', Km' Sm', Cm'	Yes	JH6
	pFK14	26	Em', Sm', Cm'	Yes	HK187

Species	Plasmid	Mol wt (x 10^6)	Related phenotype(s) *	Conjugative	Original host
	p1	36	Cryptic	?	HK187
	p2	31	Cryptic	?	HK187
	p4	4	Cryptic	?	HK187
	pDR1	45	Sm', Km'	Yes	EBC-22
	pCS2	18	Em'	?	CS14
	pCS3	19	Em'	?	CS19
	pCS4	13	Em'	?	CS20
	pCS8	13	Cryptic	?	CS29
	pCS9	26	Em'	?	CS29
	pR1401	30	Tc'	Yes	M439
	pR1402	41	Em'	Yes	M439
	pR1404	37	Tc'	Yes	M403
	pR1405	17	Em'	Yes	M440
	pX1401	39	Hly-Bac, UV'	Yes	X-14
	pX14-2	3.6	Cryptic	?	X-14
	p1P683	44	Cm', Gm'v, Km'	Yes	D366
	p1P685	20	Tc'	Yes	D366
	pCF-10	35	Tc', PR	Yes	SF-7
S.agalactiae	p1P501	20	Em', Cm'	Yes	B96(B6101)
	p1P612	23	Em', Cm'	Yes	B97(B6105)
	p1P635	20	Em', Cm'	Yes	B98
	p1P639	18	Em'	Yes	B110
	p1P640	18	Em'	Yes	B113
	p1P642	18	Em'	Yes	B115
	pMV103	18	Em'	Yes	D25303
	pMV141	17	Em'	Yes	MV141
	pMV158	3.5	Tc'	No	MV158
	pMV163	3.6	Tc'	No	MV163
	pMV120	30	Tc'	Yes	MV120
	pPB2	16	Em', Cm'	?	PB2
	pB96	17	Em', Cm'	?	B96
S.pyogenes	pAC1 (pDC10535)	17	Em'	Yes	AC1(10535)
	ERL1	19	Em'	Yes	13234
	pSM19035	18	Em'	No	19035

Species	Plasmid	Mol wt x 10^6	Related phenotype(s) *	Conjugative	Original host
	pSM22095	18	Em'	No	22095
	pSM15346	19	Em'	Yes	15346
	pSM10419	15	Em'	?	10416
S.mutans	pAM7	3	Cryptic	?	LM7
	pVA380	2.4	Cryptic	?	V380 (S.ferus)
	pVA380-1	2.9	Cryptic	?	V380 (S.ferus)
	pVA310	3.6	Cryptic	?	V310
	pVA318	3.6	Cryptic	?	V318
	pVA403	3.6	Cryptic	?	V403
S.sanguis	pAM77	4.5	Em'	No	A1
S.pneumoniae	pDP1	2.0	Cryptic	?	D39S
S.Lactis	pLM3001	30	Lac,Prt,Asa', Asi',Cr'	?	C2
	pSK04	40	Lac, Prt	?	C10
	pSK08	33	Lac, Prt	?	ML3
	pSK13	45	Lac, Prt	?	M18
	pDR1	32	Lac	?	DR1215
	pDR2	29	Suc	?	DR1215
S.cremoris	pLM3601	36	Lac	?	B1
S.diacetylactis	pGK0551	5.5	Cit	?	18-16
	pGK4101	41	Lac	Yes	18-16
	pGK0552	5.5	Cit	?	DRC1
	pGK4102	41	Lac, Prt	?	DRC1

* Abbreviations:
Tc, tetracycline; Em, erythromycin; Sm, streptomycin; Km, kanamycin; Nm, neomycin; Gm, gentamicin; Cm, chloramphenicol; Asa, arsenate; Asi, arsenite; Cr, chromate; UV, ultraviolet light; Hly-Bac, hemolysin-bacteriocin; Bac, bacteriocin; PR, pheromone response; Lac, lactose utilization; Prt, protease production; Suc, sucrose utilization; Cit, citrate utilization;', resistance

(From Clewell, D. B. (1981a), Microbiological Reviews, 45(3): 409-436)

lists a number of the plasmids isolated from members of the genus. There is obviously a great diversity in plasmid function and size. Most of the plasmids are less that 40 Md.

1.2.4. Plasmid Transfer Systems in Streptococci

All three methods of plasmid transfer, namely, transformation, transduction and conjugation have been demonstrated in the genus Streptococcus.

(a) Transformation

The phenomemon of plasmid transformation was first demonstrated in 1976 (Le Blanc, D. & Hassell, F.P., 1976), when the plasmid pAMβ1 from the DS5 strain of Streptococcus faecalis was introduced into Group F and Group H streptococci. Transformation has been reported in members of streptococcal Groups F, H, N and O (Dobrzanski, W.T., 1972; Perry, D. & Slade, H.D., 1962; Westergren, G., 1978), Streptococcus mutans (Perry, D. & Kuramitsu, H.K., 1981), and Streptococcus pneumoniae (Lacks, S.A., 1977; Tomasz, A., 1969).

Transformation occurs in Streptococcus sangius and Streptococcus pneumoniae only at a specific stage in logarithmic growth when an optimal cell density is achieved. At this stage in growth the micro-organism is said to be in a competent state, and recent experiments have demonstrated that both Streptococcus pneumoniae (Morrison, D.A. & Baker, M.F., 1979) and Streptococcus mutans (Raina, J.L. & Ravin, A.W., 1980) induce the synthesis of a group of proteins when the micro-organism is moving towards the competent state. Lacks (Lacks, S.A., 1977; Lacks, S., 1979) has

reported that in Streptococcus pneumoniae only a single DNA strand enters the new host cell during transformation. This is because the covalently closed circular DNA, becomes nicked whilst binding to the cell surface of the new host and the complementary DNA strand is degraded. Deletions in the plasmid DNA have been shown to occur whilst the plasmid is establishing itself in the host cell (Behnke, D. et al., 1979; Le Blanc, D. & Hassell, F.P., 1976; Shoemaker, N.B. et al, 1979). Clewell (Clewell, D., 1981a) suggests that there is probably plasmid circularization difficulties when a plasmid enters a new host cell as presumably there is a lack of internal homology, and it may be because of this that deletions occur. Monomeric forms of plasmid DNA transform Streptococcus sanguis and Streptococcus pneumoniae much less efficiently than the Streptococcus pneumoniae much less efficiently than the oligomeric forms (Macrina, F.L. et al., 1918; Saunders, C.W. & Guild, W.R., 1981). There have been several reports of plasmid transformation between different species of streptococci and between different genera (Davidson, J.R.Jr. et al., 1976; Pakula, R. et al., 1959, Perry, D. & Slade, H.D., 1962; Westergren, G. & Emilson, C.G., 1977).

(b) Transduction

Transduction via a bacteriophage has been demonstrated in streptococcal Groups A, C, G and N (Colon, A.E. et al., 1972; Hyder, S.L. & Stretfeld, M.M., 1978; Wannamaker, L.W. et al., 1973; McKay L.L. et al., 1976). This mode of gene transfer has also been shown to occur in pneumococci, (Porter, R.D. et al., 1979). Bacteriophage-mediated transduction has not been shown to occur in streptoccal Groups B and D.

(c) Conjugation

In 1974 Jacob and Hobbs (Jacob, A. & Hobbs, S.J., 1974) demonstrated conjugal transfer of a plasmid encoding multiple drug resistance from the JH1 strain of Streptococcus faecalis using a broth mating technique. Subsequent experiments eliminated the possibility of transfer via transduction or transformation thus indicating transfer requiring cell-to-cell contact.

Conjugative plasmids have also been shown to mobilise non-conjugative plasmids (Burdett, V., 1980; Dunny, G. et al., 1979; Oliver, D. et al., 1977; Smith, M.D., 1980; Tomich, P., 1979) and chromosomal markers (Frank, A. & Clewell, D.B., 1981; Franke, A. et al., 1978).

The conjugative plasmid pAMβ1 from Streptococcus faecalis strain DS5 (Clewell, D. et al., 1974) which codes for erythromycin resistance has been shown to be highly promiscuous, since it exhibits a wide range of hosts. This plasmid has been shown to transfer into nine different species of streptococci and it is also capable of intergenic transfer. In Streptococcus faecalis inhibition of transfer of pAMβ1 has been observed if the plasmid pAMγ1 or pAD1 is also present in the donor strain (Clewell, D., 1981a).

The plasmid pAMβ1 belongs to a group of plasmids known as MLS plasmids. These plasmids exhibit resistance to macrolides, lincosamides, and streptogrammin B. These types of plasmids have

been demonstrated in many Lancefield Groups and many are capable of intra-species and interspecies tranfer.

A recent study of nine conjugative drug resistance plasmids (Horodniceanu, T. et al., 1982a) from four strains of Streptococcus faecalis (Group D) two strains of Streptococcus faecium (Group D) and one strain of Streptococcus agalactiae (Group B) using recipients from many of the streptococcal Lancefield Groups as well as strains of different genera, has shown that drug resistance plasmids from Streptococcus faecalis and Streptococcus faecium display a narrow host range. Plasmid DNA was only demonstrated after intra-species conjugation except in two cases of Group B Streptococcus recipients. The host range of the plasmid harboured by Streptococcus agalactiae had a wide host range. Horodniceanu and co-workers conclude that streptococcal resistance plasmids can be divided into two groups. One group contains the macrolide resistance plasmids harboured by different species of streptococci. These plasmids exhibit a broad host range and a very similar restriction endonuclease pattern. The other group is composed of tetracycline and aminoglycoside resistance plasmids present in the enterococcal species which display a narrow host range and a different restriction endonuclease pattern.

The conjugative plasmids found in strains of Streptococcus faecalis can be divided into two groups (Clewell, D., 1981a). Plasmids which are capable of transferring their markers at a high frequency (10^{-3} to 10^{-1} transconjugants per donor) in broth mating and plasmids which exhibits low frequency of transfer,

(often less than 10^{-6} transconjugants per donor) in broth mating, with an increased frequency of transfer (10^{-4} to 10^{-2} transconjugants per donor) when mated on filter membranes. Clewell (Clewell, D., 1981a) has demonstrated that strains of Streptococcus faecalis harbouring plasmids capable of a high transfer frequency in broth mating use pheromones to achieve cell-to-cell contact, whilst those with a low frequency to transfer do not produce pheromones. As mentioned previously (1.1.2) these pheromones are small peptides with a molecular weight of less that 1,000. They are protease-sensitive, soluble, heat-stable molecules released by recipient cells. A specific phermone is defined by the plasmid system used to resolve it and the pheromones cAD1 (from strains harbouring the plasmid pAD1) and cPD1 (from strains harbouring the plasmid pD1) have been purified and sequenced (Mori, M. et al., 1984; Suzuki, A. et al., 1984). Both pheromones are very hydrophobic octapeptides. Pheromones induce donor cells to produce a proteinaceous adhesive substance known as aggregation substance (AS) (Ike, Y & Clewell, D.B., 1984). They also induce specific donor cells to adhere to the recipient thus forming an aggregate which facilitates the passage of plasmids from donor to recipient (Clewell, D., 1981b). The pheromone is also referred to as a " clumping inducing agent" (CIA), since an aggregation response can also be demonstrated in a cell free filtrate of the recipient strain when mixed with donors. The clumping phenomenon usually required 30 to 45 minutes before aggragation can be seen. The frequency of transfer of plasmid can be markedly elevated by mixing the recipient filtate with donor cells for 20 to 50 minutes prior to a 10 minute mating.

Once the recipient cell has received the conjugative plasmid it cease to produce the CIA specific to that plasmid, however it does respond to the production of CIA by other recipients cells not harbouring the plasmid. The "shutting off" of endogenous pheromone subsequent to the aquisition of a plasmid has been investigated by Ike and co-workers (Ike, Y et al., 1983). They have found that donor cells harbouring either the 37.8 Md plasmid, pADl, or the 36.4 Md plasmid pPDl, produce a substance which corresponds to a modified inactive form of the related pheromone. This modified pheromone has a mass 350 to 450 daltons larger than the active pheromone and when treated with phosphodiesterase II, pheromone activity was restored. Donors containing different conjugative plasmids respond to different pheromones (Dunny, G. et al., 1979) and recipients are able to produce a number of different pheromones. Clewell (Clewell, D., 1981a) suggests that "the pheromone induces a polycistronic operon (perhaps somewhat analogous to the Tra operon of certain conjugative plasmids in gram-negatve bacteria) which in addition to having determinants related to aggregation, also determines functions related to transfer".

Genetic analysis of the pADl phermone response in Streptococcus faecalis using deletion mutants generated by the insertion of the erythromycin resistance transposon, Tn917 into the pADl plasmid revealed that the mutants which exhibited constitutive clumping (due to the production of AS) and a high frequency of transfer of erythromycin resistance, fell into two distinct sub-classes. These sub-classes were recognizable by

their different morphologies. These two types of morphology were classified as "dry" and "normal". Restriction enzyme analysis allowed the mapping of the Tn917 insertions to two discrete clusters designated tra A and tra B. The tra A cluster constituted the "dry" colony subclass and tra B the "normal" colony. Constitutive clumpers were shown not to produce the phermone cAD1 associated with the plasmid pAD1. Ike and colleagues believe that these results suggest a form of negative control of the proteinaceous adhesin (AS).

Dunny and co-workers (Dunny, G. et al., 1979) have demonstrated that the production of phermone by recipient cells, which are plasmid free, levels off or is drastically reduced when the cells enter the stationary phase of growth. The decrease is probably due to the action of a gelatinase which is present in these strains. Studies using a mutant strain, which does not produce gelatinase, have shown that in the absence of gelatinase no decrease in the production of phermone is observed in the stationary phase (Clewell, D., 1981a).

Dunny and co-workers (Dunny, G. et al., 1979) have also shown that drug sensitive strains of streptococci are less able to respond to and produce clumping inducing activities than strains carrying single and multiple drug resistance.

It is of interest to note that the phermone cAM373 produced by the Streptococcus faecalis strain RC73 which harbours the conjugative plasmid pAM373, has recently been shown to be produced by a number of strains of Staphylococcus aureus as well

as a Streptococcus faecium strain and two Streptococcus sanguis strains (Clewell, D.B. et al., 1985). Clewell suggests that although the other bacterial species excrete a substance resembling the Streptococcus faecalis sex phermone cAM373 this does not necessarily indicate that these substances are sex phermones in these species, since pAM373 transconjugants could not be produced in Staphylococcus aureus or Streptococcus sanguis suggesting that cAM373 was not directly involved in mating. Clewell concludes that, "it is conceivable that bacterial production of the substances which donor strains recognise as sex phermones could have preceded the evolution of the related conjugative plasmid systems with the latter simply taking advantage of the available, extracellular compounds as mating signals".

Transfer of the drug resistance in the absence of plasmid DNA has been demonstrated in Streptococcus faecalis (Horodniceanu, T. et al., 1982a; Franke, A. & Clewell, D., 1980; Franke, A. & Clewell D.B., 1981b) (see section 1.2.5) Streptococcus pneumoniae (Buu-hoi, A. & Horodniceanu, T., 1980; Shoemaker, N. et al., 1979) (see section 1.2.5), Streptococcus faecium (Le Bougenec, C. & Horodniceanu, T. et al., 1982b), Streptococcus bovis (Horodniceanu, T. et al., 1982b), Streptococcus milleri (Horodniceanu, T. et al., 1982b), Streptococcus mutans (Le Blanc, D., 1981) in Group A, B, F, H, and G (Smith, M.D. & Guild, W.R., 1982; Horodniceanu, T. et al., 1981; Hawley, R.J. et al., 1980). The plasmid-free transfer to resistance markers from donor to recipient requires cell-to-cell contact, so Clewell has suggested that the determinants may

reside on "conjugative transposons" (Clewell, D.B., 1981a).

1.2.5 Tetracycline resistance determinants in the Genus Streptococcus

In general, tetracycline resistance in streptococci does not appear to be plasmid borne. A survey of tetracycline resistant Group B streptococci (Burdett, V., 1980), revealed that only 3 isolates out of 30 contained plasmid DNA. Another survey of tetracycline reisitant streptococci (Hawley, R.J. et al., 1980) mainly Group H strains isolated from the human oral cavity demonstrated plasmid DNA in only 23 out of 121 isolates.

A tetracycline and chloramphenicol resistance determinant in a strain of Streptococcus pneumoniae has been shown, by transformation, to be chromosomally linked (Shoemaker, N.B. et al., 1979). Conjugal transfer of the tetracycline resistance to a strain of the same species was achieved via a filter mating procedure although no plasmid DNA could be detected in either the donor strain or the transconjugants.

Tetracycline resistance encoded by a transposon, Tn916, in Streptococcus faecalis has been shown to be capable of "conjugal" transfer (Franke, A.E. & Clewell, D.B. 1981). This transposon does not require the presence of a conjugative plasmid in order to transfer. The transposon is capable of transposing to a number of conjugative plasmids. Clewell suggests that Tn916 could be a plasmid-like or phage-like element that is unable to replicate on its own but has sufficient genetic information to enable the transposon to transfer. This transfer could be

achieved by three different means:- duplication of the transposon, excision of the element or both duplication and excision. The next step could then be expression of the transfer functions and once the DNA had transferred to the recipient an "integrase" could be "zygotically induced" which would allow insertion of the transposon into the bacterial chromosome. Gawron-Burke (Gawron-Burke, C. & Clewell, D.B., 1982) has demonstrated that multiple copies of Tn916 are generated in some transconjugants. She suggests that this could be a result of secondary transposition to an unreplicated area of the chromosome from a replicated area. Once Tn916 enters a new host a period of time may need to elapse before the transposon is sufficiently stable to be maintained at one site.

Recently it was attempted to clone the transposon Tn916 on to a plasmid vector in Escherichia coli in order to facilitate the genetic analysis of this transposon (Gawron-Burke, C. & Clewell, D.B., 1984). However, Tn916 was found to be unstable in the recombinant plasmid and was excised at a high frequency when not grown in the presence of tetracycline. Transformation of Streptococcus sanguis (Challis) to tetracycline resistance by the recombinant resulted in the excision of Tn916 from the plasmid followed by insertion of the transposon into the chromosome of the recipient. The results indicate a means for the cloning and targeting of genes from gram-positive bacteria,since Tn916 could be used as a "convenient mutagen via its transpositional insertion into plasmid DNA or its conjugative insertion into the chromosome of recipient bacteria"(Gawron-Burke,C.& Clewell,D.B.1984)

Two conjugative transposons encoding tetracycline resistance have recently been described. These two transposons designated Tn918 and Tn919 were found in Streptococcus faecalis strain RC73 (Clewell, D.B. et al., 1985) and Streptococcus sanguis strain, FC1 (Fitzgerald, G.F. & Clewell, D.B., 1985), respectively. Both transposons have been shown to bear considerable homology to Tn916. This would seem to suggest a common origin for these three transposons, and since Tn916 is capable of transfer into a number of different gram-positive species it is probable that these transposons are widespread.

A study of a tetracycline resistance determinant found in a porcine isolate of Streptococcus mutans, strain DL5, has also been shown to exhibit transfer of tetracycline resistance in the absence of plasmid DNA (Le Blanc, D., 1981). Examination of tetracycline resistant transconjugants resulting from mating DL5 with Streptococcus faecalis strain JH2-2 and a recombination deficient strain of Streptococcus faecalis UV202, revealed the presence of an 8 Md plasmid not present in the donor strain. However these tetracycline resistant transconjugants were unable to transfer their resistance. When DL5 was mated with Streptococcus mutans DR0001/1, and the transconjugants examined, a plasmid band of 19 Md was demonstrated occasionally. Transconjugants from this latter mating were able to transfer tetracycline resistance. Le Blanc suggests that this system is similar to that found in many antibiotic resistant strains of Haemophilus influenzae (Roberts, M.C. & Smith, A.L., 1980; Stuy, J.H., 1980), in which the conjugative plasmids appear to have integrated into the chromosome. At present, Le Blanc and

co-workers are endeavouring to isolate enough plasmid DNA from the transconjugants to prepare labelled probes and therefore ascertain whether chromosomal integration of plasmid DNA is taking place in the Streptococcus mutans strains.

An analysis of naturally occurring tetracycline resistance in streptococci, (Burdett, V. et al., 1982a,b) using deletion analysis and DNA:DNA hybridization techniques, has shown that at least three genetic determinants are responsible for tetracycline resistant phenotype. The tet L determinant was located on small non-conjugative plasmids and mediated tetracycline resistance. Deletion analysis and DNA:DNA hybridization suggests that the determinant has a size of 1,850 base pairs. The tet M determinant was not usually associated with a plasmid and conferred resistance to minocycline as well as tetracycline. The tet M determinant was cloned from the total cellular DNA of Streptococcus agalactiae strain B109 and this strain is able to transfer tetracycline resistance in the absence of plasmid DNA. A recombinant plasmid designated pJ12 was constructed containing the tet M determinant cloned from total cellular DNA of B109 as a chimera with the Escherichia coli plasmid pVH2124 and analysis of pJI2 revealed that the tet M determinant was located within a 5.0 kilobase region of the hybrid. The tet N determinant was found on a large conjugative plasmid, pMV120, form Streptococcus agalactiae and it showed no homology with the tet M to tet L genetic probe (Table 3).

Radioactively labelled probes for the cloned tetracycline resistance determinants tet L and tet M were used in colony hybridization studies to investigate the relative frequency of

TABLE 3

Summary of Southern hybridization data

Sources of DNA electrophorsed on gel	Radioactive probe DNA	
	pVB.A15	pJI2
Tc^r plasmids		
pMV158, pMV163, pAM1	±	-
pMV120	-	-
pAM211, pIP614	-	±
Tc^r S.agalactiae		
B109, MV107, MV158, MV159, MV160, MV206	-	±
Tc^r S. faecalis		
DS16p	-	±
Tc^r S pneumoniae		
N77	-	±
Tc^r Streptococcus spp.		
$JH2\text{-}2_d$, $MV762_B$	-	±

The two genetic probes represented by pVB.15 (pMV158 from Streptococcus agalactiae as a chimera with Escherichia coli plasmid pVH2124), and pJ12 (total cellular DNA from Streptococcus agalactiae strain B109 as a chimera with the Escherichia coli plasmid pVH2124), specify the tetracycline resistance determinants tetM and tetL respectively.

(From Burdett, V. et al (1982), Journal of Bacteriology 149: 995-1004)

these determinants in nature.The results revealed that these two determinants are widespread.Indeed,31 out of the 31 human and animal isolates of Streptococcus faecalis(containing 2 to 6 plasmids and resistant to more than one drug)reacted with the tet L and 30 with the tet M probe(Burdett,V. et al.,1982b).However,out of 13 clinical isolates of Streptococcus agalactiae investigated(containing few plasmids and infrequent resistance to more than one drug),12 reacted with tet M,two with tet L and one with neither.Thus, it would appear that tet M-like determinants are widespread in nature.

Strains carrying tet L, tet M or tet N alone exhibit constitutive expression of tetracycline resistance and this coupled with the genetic differences between these tetracycline determinants may reveal that the mechanism of tetracycline resistance in the streptococci differs from the tetracycline resistance mechanism of other micro-organisms which express inducible resistance, supposedly via an alteration in tetracycline transport. Indeed recent work by Burdett has demonstrated differences in the accumulation of tetracycline between different tetracycline resistance determinants suggesting variation in the mechanisms of tetracycline resistance between these different determinants (Burdett, V., 1985).

Work on the Streptococcus faecalis strain JH1, has revealed that two different genetic determinants mediating tetracycline resistance are present in this strain (Le Blanc, D.J. & Lee, L.N., 1982). One determinant located on plasmid pJH1 mediates constitutive tetracycline resistance. The second determinant, which is either chromosome-borne or located on an undetectable

plasmid, mediates an inducible tetracycline resistance.

These two determinants showed no sign of homology when subjected to DNA:DNA hybridization. Burdett has shown, using colony hybridization techniques that the tetracycline resistance determinant located on the plasmid pJH1 shows homology with the tet L determinant (Burdett, V. et al., 1982). This finding is surprising since pJH1 is a large conjugative plasmid and studies, to date, indicate that the tet L determinant appears to be associated with small non-conjugative plasmid.

Le Blanc and Lee (Le Blanc, D.J. & Lee, L.N., 1982) also found that one transconjugant, DL172, produced by mating Streptococcus faecalis JH1, was resistant to 80μg ml^{-1} of tetracycline, sensitive to kanamycin, streptomycin and erythromycin and haemolytic only in the presence of tetracycline. A single plasmid pDl172, was isolated from the transconjugant and it was found to be composed of plasmid pJH2 and a 17.8 kilobase fragment of DNA homologous to the total cell DNA of strain JH1 however, pDL172 did not contain pJH1. Experiments are in progress to investigate whether or not the heterologous DNA was added to pJH2 via the translocation of a 17.8 kilobase transposon or by classical recombination with pJH2. The degree of homology between the 17.8 kilobase segment and the tetracycline resistance transposon Tn916 in Streptococcus faecalis strain DS16 (Franke, A.E. & Clewell, D.B., 1980) is also being investigated.

Banai and Le Blanc (Banai, M. & Le Blanc, D.J., 1983) have constructed a restriction endonuclease map of plasmid pJH1 and they have reported that "a number of multiple antibiotic

resistance plasmids harboured by Streptococcus faecalis strains isolated from human patients and from farm animals share considerable DNA:DNA homology with pJH1".

The tetracycline resistance plasmid pAMα1 (Clewell, D. et al., 1975) has been shown to be a composite of two separable replicons (Perkins, J.B. & Youngman, P., 1983). One of these replicons has been shown to be closely related to the tetracycline containing Bacillus cereus plasmid pBC16. Perkins and Youngman suggest that the high conservation of homology between these two plasmids indicates that the selective pressure for the dispersal of this common tetracycline determinant is possibly very recent.

1.2.6 Objectives of this survey

The aim of this present work is to carry out a survey of genetic element responsible for tetracycline resistance in Group D streptococci as no survey has been carried out on tetracycline resistance in this Group. Surveys have been carried out on tetracycline resistant strains of Group B streptococci (Burdett, V., 1980) and revealed plasmid DNA in only 3 out of 30 strains. A survey or oral streptococci (Hawley, R.J. et al., 1980) also revealed plasmid-less tetracycline resistance.

The frequency of transfer of tetracycline resistance using the JH2-2 strain of Streptococcus faecalis (Jacob, A. & Hobbs, S.J., 1974) as the recipient strain will be investigated and the plasmid content of both the parental and transconjugant strains examined. Following this, the sex factor pAMβ1 (Clewell, D. et

al., 1974) will be introduced into the donor strains which will then be mated with the JH-2-2 strain to see if the frequency of transfer of the tetracycline determinant has changed. the tetracycline resistant transconjugants from the sex factor matings will also be examined for plasmid DNA content, to see if the plasmids present in the original tetracycline resistant donor strains have been mobilized.

A series of experiments will be performed to investigate whether or not plasmids present in the parental and transconjugant strains do encode the tetracycline resistance determinant. The degree of homology between the types of determinant disclosed by this survey and other reported tetracycline resistance determinants (Burdett, V., 1982) will also be investigated. This should provide a valuable comparison between determinants characterized in the United States and those carried by streptococcal strains in Britain as it will indicate the presence of DNA sequences conserved during the evolution of the various types of determinant.

2. MATERIALS AND METHODS

2. MATERIAL AND METHODS

2.1 Bacterial strains

The tetracycline resistant strains of the Genus Streptococcus, all of which have a minimum inhibitory concentration for tetracycline of 64 μg ml^{-1} higher are show in table 4. These strains were provided by Dr. D. Thirkell and Dr. M. Blankson of St. Andrews University. The strains originate from two sources, sewage and clinical isolates. The erythromycin resistant strain SSA13, containing the sex factor pAMβ1, and DS5(Clewell,D.B. et al.,1974) were both provided by Sheila Unkles also of St. Andrews University. The JH strains were provided by Dr. A. Jacob of the University of Manchester, Medical School. Strains of Escherichia coli harbouring recombinant plasmids were provided by Dr. Vickers Burdett of Duke University, U.S.A. These two strains of Escherichia coli are designated SK1592 (pVB.A15) and SK1592 (pJI3)(Burdett,V. et al.,1982,a.b).

2.2 Media for the mating procedure

2.2.1. N2TG

The nutrient broth used for growing streptococcal strains was N2TG, and is composed of:

	grams $litre^{-1}$	
Lab-lemco powder (Oxoid)	10	g
Peptone (Oxoid)	10	g
Sodium Chloride (BDH)	5	g
Tris(hydroxymethylaminomethane) (Sigma)	12.1	g
Glucose (Boots Co. Ltd.)	2	g
Distilled water	950	m

TABLE 4

Bacterial Strains used in this Study

STRAIN	Species	M.I.C. for tetracycline ug ml^{-1}	Phenotype
K4	S. faecalis var. faecalis	64	
K22	S. faecalis var. faecalis	256	
K37	S. faecalis var. faecalis	64	
K42	S. faecalis var. liquefaciens	64	
K45	S. faecalis var. faecalis	64	
K47	S. faecalis var. faecalis	64	
K48	S. faecalis var. faecalis	64	
K52	S. faecalis var. faecalis	64	
K54	S. faecium	64	
K57	S. faecalis var. liquefaciens	64	
K58	Unidentified Group D Streptococci	64	
K59	S. faecalis var. faecalis	64	
K65	S. faecalis var. faecalis	64	
K76	S. faecalis var. faecalis	128	Tc^r, fus^s, rif^s
K78	S. faecalis var. liquefaciens	128	
K89	S. faecalis var. faecalis	64	
K91	S. faecalis var. faecalis	128	
K93	S. faecalis var. faecalis	128	
K97	S. faecalis var. faecalis	64	
SB38	S. faecalis var. faecalis	64	
SB62	S. faecium	64	
SB65	S. faecium	64	
SB68	S. faecium	64	
SB74	S. faecalis var. faecalis	64	
SB77	S. faecalis var. faecalis	64	
SB79	S. faecalis var. faecalis	64	
SB80	S. faecalis var. faecalis	64	
SB88	S. faecium	64	
SA13	S. faecalis var. liquefaciens	64	
SA92	S. faecalis var. liquefaciens	64	
JH1-18	S. faecalis	-	fus^r, bac-hem
JH1-23	S. faecalis	-	
JH2	S. faecalis	-	rif^r, Sm^r, Thy
JH2-18	S. faecalis	-	Tc^s, fus^r, rif^r
JH2-2	S. faecalis	-	Tc^s, Em^r, fus^r, rif^r
SSA13	S. faecalis	-	
JH1-2	**S. faecalis**		**Em^r, Tc^r, Km^r, Nm^r, Gm^r, Thy**

Abbreviations:

1. K - clinical isolate from Victoria Hospital, Kirkcaldy.
2. SB - isolate from below the sewage out flow.
3. SA - isolate from above the sewage out flow.
4. r - resistant, s - sensitive, Tc - tetracycline, fus - fusidic acid, rif - rifampin, Em - erythromycin, bac-hem - bacteriocin hemolysin producing, Sm - streptomycin, Thy - Thymine requiring.

NOTE: Strains K4 to SA92 were sensitive to ampicillin, penicillin, gentamicin, streptomycin and erythromycin. They were also non-haemolytic. All strains with the exception of strain K76 were also sensitive to chloramphenicol.

The media was made up as indicated by the manufacturers. The pH was adjusted to 7.8 with 5M HCl and the final volume of the medium was made up to 1 litre, and then sterilized for 15 minutes at 115°C (10 pounds square inch^{-1} steam pressure).

2.2.2. DST

The agar used was DST (Oxoid) which consists of the following formula:

grams litre^{-1}		
Protease peptone (Oxoid)	10	g
Veal infusion solids	10	g
Dextrose	2	g
Sodium chloride	3	g
Disodium phosphate	2	g
Sodium acetate	1	g
Adenine sulphate	0.01	g
Guanine hydrochloride	0.01	g
Uracil	0.01	g
Xanthine	0.01	g
Aneurine	0.000002	g
Agar No. 1 (Oxoid)	12 g	

pH 7.4 approx.

The agar was supplemented with 2 g litre^{-1} of glucose (Boots Co. Ltd.). One litre of the medium was made up by disolving 40 g of DST agar powder and 2 g of glucose in 1 litre of distilled water and boiling the solution until the agar dissolved. The medium was sterilized for 15 minutes at 115°C (10 pounds aquare inch^{-1} steam pressure). The medium was allowed to cool before the addition of antibiotics.

2.3. Antibiotics for the mating procedure and curing

Antibiotics were used in the DST agar plates at the following final concentrations: Streptomycin (Sm) (Sigma) 2000 μg ml^{-1}; Tetracycline (Tc) (Sigma) 20 μg ml^{-1}; fusidic acid (Fus) (Leo Laboratories Ltd. 25 μg ml^{-1}; choramphenicol (Cm) (Sigma) 20 μg ml^{-1} erythromycin (Em) (Sigma) 20 μg ml^{-1}; rifampin (Rif) (Calbiochem-Behring Corp.) 100 μg ml^{-1}; Novobiocin (Sigma) was used at various concentrations.

2.4. Media for plasmid isolation and curing

2.4.1.(a) Brain Heart infusion broth and agar

Brain heart infusion (BHI) (Oxoid) was prepared as indicated by the manufacturers. Brain Heart infusion broth plates were supplemented with 1%(w/v) bacteriological agar No. 1 (Oxoid). Thymus was added at 20 μg ml^{-1} when required.

2.4.1.(b) BYGT broth supplemented with threonine and tetracycline

The broth for growing up the bacterial strains was composed of the following:

	grams $litre^{-1}$	
Brain heart infusion broth powder (Oxoid)	19	g
Yeast extract (Difco Labs.)	5	g
Glucose (Boots Co. Ltd.)	2	g

To the above constituents was added: 0.1 volume of 1.0M of Tris(hydroxymethylaminomethane) pH 8.0, 20 mM DL-threonine (BDH) (4.76 g litre),and 20 μg ml^{-1} tetracycline. The final volume of the medium was made up to 1 litre with distilled water.

The medium was then autoclaved at 115°C for 15 minutes at (10 pounds square inch^{-1} steam pressure). The medium was stored at 4°C.

2.4.1.(c) L-Broth

	grams litre^{-1}	
NaCl	10	g
Tryptone (Difco)	10	g
Yeast extract (Difco)	5.0	g

Distilled water was added to a final volume of 1000 ml. The medium was autoclaved at 115°C for 15 minutes (10 pounds square inch^{-1} steam pressure). The medium was stored at 4°C.

2.4.2. Stock solutions

(a) 1M Tris(hydroxymethylaminomethane) (Tris)

This stock solution was made by dissolving 121.41 g of Tris in distilled water, adjusting the pH to 8.0 with 10 M HCl, and adding more distilled water to give a final volume of 1 litre. The solution was stored at 4°C.

(b) 0.25 M Ethylenediaminetetraacetic acid (EDTA)

This stock solution was made by dissolving water, adjusting the pH to 8.0 with 10 M NaOH, and adding more distilled water to give a final volume of 1 litre. The solution was stored at 4°C.

(c) 5 M Sodium chloride (NaCl)

This stock solution was made by dissolving 146 g of NaCl in 1 litre of distilled water. The stock solution was stored at

$4^{o}C$.

2.4.3. Buffer solutions

The buffers (a) and (b) were made from the stock solutions in section 2.4.2.

(a) TE buffer (50 mM Tris, 5mM EDTA, pH 8.0)

	volume l^{-1} of the stock solution	
50 mM Tris	50	ml
5 mM EDTA	20	ml

Distilled water was added to the above solutions, the pH adjusted to 8; and the final volume of the buffer was made up to a litre with distilled water.

(b) TE buffer (10 mM Tris, 1 mM EDTA pH 8.0)

	volume l^{-1} of the stock solution	
10 mM Tris	10	ml
1 mM EDTA	4	ml

Distilled water was added to the above solutions, the pH adjusted to 8.0 and the final volume made up to 1 litre with distilled water. The buffer was stored at $4^{o}C$.

(c) TE (10) buffer (10 mM Tris, 10 mM EDTA, pH 8.0)

	volume l^{-1} of the stock solution	
10 mM Tris	10	ml
10 mM EDTA	40	ml

Distilled water was added to the above solutions, the pH

adjusted to 8.0 and the final volume made up to 1 litre with distilled water. The buffer was stored at $4^{o}C$

(d) TES buffer (50 mM Tris, 5mM EDTA, 1M NaCl, pH 8.0)

	volume l^{-1} of the stock solution	
50 mM Tris	50	ml
5 mM EDTA	20	ml
1.0 M NaCl	200	ml

Distilled water added to the above solutions, the pH adjusted to 8.0 and the final volume made up to 1 litre with distilled water. The buffer was stored at $4^{o}C$.

(e) TBE buffer

	grams litre (10x)		grams litre (1x)	
Tris	108.0	g	10.8	g
EDTA	9.3	g	0.93	g
Boric acid powder (May & Baker)	55.0	g	5.5	g

The reagents were dissolved in distilled water, the pH adjusted to 8.1, and the final volume made up to 1 litre was distilled water. The buffer was stored at $4^{o}C$.

2.4.4. 3 M Sodium acetate (NaAc)

This was prepared by dissolving 24.6 g of analar NaAc (BDH) in 100 ml of distilled water. The solution was stored at $4^{o}C$.

2.4.5. 3 M Potassium acetate (KAc)

Potassium acetate (BDH) solution was prepared by dissolving 24.6 g of potassium acetate in 50 ml of distilled water. The solution was stored at $4^{o}C$.

2.4.6. 2 M Tris (hydroxymethylaminomethane) (Tris)

This was prepared by dissolving 24.428 g of Trizma base in distilled water, adjusting the pH to 8.0, and bringing the final volume to 200 ml with distilled water. The solution was stored at $4^{o}C$.

2.4.7. Lysozyme

Lysozyme (Sigma) solution was prepared by dissolving the enzyme in TE buffer to give a final concentration of 40 mg ml^{-1}.

2.4.8. Proteinase K

Proteinase K (Boehringer Mannheim) was prepared by dissolving the enzyme in distilled water to give a final concentration of 10 mg ml^{-1}.

2.4.9. Sodium dodecyl sulphate (SDS)

SDS (Sigma) was prepared at a concentration of 1%(w/v), by dissolving 0.1 g of SDS in 10 ml of TE buffer and adjusting the pH to 12.45 with 10 M NaOH immediately before use.

2.4.10. Agarose

Agarose (Bethesda Research Labs.) was prepared at various concentrations by boiling the agarose in 1 x TBE buffer in order to dissolve it.

2.4.11. Bromophenol blue dye for Gel Electrophoresis

This dye was made by dissolving 0.5g of bromophenol blue powder(BDH) and 0.5g of xylene cyanol FF (BDH) in 50ml of distilled water.Once dissolved,the volume was made up to 100ml with 50ml of glycerol(BDH).The solution was stored at $4^{o}C$

2.4.12. Ethidium bromide

A solution of ethidium bromide (Sigma) was prepared by dissolving 1 g of ethidium bromide in 100 ml of distilled water to give a final concentration of 10 mg ml^{-1}.

2.4.13. Chloroform - isoamyl alcohol (24:1v/v) extraction mixture

A volume of 500 ml of this mixture was prepared by adding 20 ml of isoamyl alcohol to 480 ml of chloroform.

2.4.14. Phenol saturated with 3% sodium chloride

One kilogram of phenol (BDH) was re-distilled and an equal volume of 3%(w/v) sodium chloride (BDH) solution was added to the distillate to produce a saturated solution.

2.5. Solutions used for the isolation of recombinant plasmids from Escherichia coli.

(a) Lysis buffer (Solution I)

1 M glucose	2.5	ml
1 M Tris -HCl, pH 8	1.25	ml
0.2 M EDTA, pH 8	2.5	ml
Lysozyme	250	mg

The buffer was made up to 50 ml final volume with distilled water and stored at 4°C.

(b) Alkaline solution (Solution II)

5 M NaOH	3.2	ml
10%(w/v) SDS	8.0	ml

Distilled water was added to give a final volume of 80 ml.

(c) Neutralisation solution (Solution III)

This solution was made by dissolving 11.8 g of potassium acetate in 30 ml of distilled water. The pH of the solution was adjusted to 5.0 with approximately 5 ml of glacial acetic acid. Distilled water was added to give a final volume of 40 ml.

(d) Phenol - chloroform - isoamyl alcohol (24:24:1v/v)

One bottle of the phenol (Analar) (250 g) was saturated with 0.1 M Tris, pH8. After separation into two layers 8-hydroxyquinoline was added to the phenol layer to give a final concentration of 0.1%(w/v). This reagent is an anti-oxidant, weak chelator and partial RNase inhibitor. Chloroform-isoamyl alcohol (24:1 v/v) was prepared and 1 volume of this was mixed with 1 volume of phenol-Tris-8-hydroxyquinoline. After equlibration most of the aqueous layer was removed and the reagent stored at 4°C.

2.6. Solutions used for Southern Blotting and DNA:DNA hybridization

(a) Solution for alkali denaturation of DNA in agarose

	grams $litre^{-1}$	
NaOH (Sigma)	20	g
NaCl	87	g

Dissolve NaOH pellets and NaCl in 1 litre of distilled water and store at room temperature.

(b) Neutralization buffer (for use after alkali denaturation)

	grams $litre^{-1}$	
1.5 M NaCl	88.2	g
1.0 M Tris	121	g

The NaCl and Tris were dissolved in 800 ml of distilled water. The pH of the buffer was adjusted to pH8 using approximately 52 mls of concentrated HCl. The volume of the buffer was adjusted to 1 litre with distilled water. Neutralization buffer was stored at room temperature.

(c) 20 x SSC

	grams $litre^{-1}$	
NaCl	175.3	g
Sodium citrate (BDH)	88.2	g

The NaCl and sodium citrate were dissolved in 800 ml of water. The pH of the solution was adjusted to pH 7 with a few

drops of 10 M NaOH and the volume adjusted to 1 litre.

(d) Prehybridization buffer

3 x SSC, 10 x Denharts

This buffer composed of the following:-

75	ml	20 x SSC
10	ml	0.5 M EDTA
2.5	ml	20%(w/v) SDS
5	ml	Denatured sonicated salmon sperm (Sigma) ($10\ mg\ ml^{-1}$) to give a final concentration of $100\ \mu g\ ml^{-1}$
1	g	Polyvinyl pyrrolidine (Pharmacia)
1	g	Ficoll (Pharmacia)
1	g	Bovine Serum Albumin (Sigma)

These constituents were dissolved in distilled water to give a final volume of 500 ml. The buffer was stored at room temperature.

(e) Hybridization buffer

This buffer is composed of prehybridization buffer containing 9%(w/v) Dextran Sulphate (Pharmacia) and it was stored at room temperature.

(f) "Blot" washing solution

	$ml\ l^{-1}$	
20 %(w/v) SDS	5	ml
20 x SSC	5	ml

The volume of this buffer was made up to 1 litre with distilled water. The buffer was made up fresh each time.

2.7. Solutions used for nick translation of DNA probes

Solutions 1,2, and 3 were components of the Amersham Nick Translation Kit (code number N.5000). The solutions contained the following:-

(a) Solution 1

100 μM dATP, 100 μM dGTP and 100 μM dTTP in a concentrated nick translation buffer solution containing Tris-HCl, pH 7.8, magnesium chloride and 2-mercaptoethanol.

(b) Solution 2

Enzyme solution - each 10 μl aliquot was composed of 5 units polymerase I and 100 pg DNase I in magnesium chloride, glycerol and bovine serum albumin.

(c) Solution 3

Carrier DNA in solution was used to precipitate labelled DNA when monitoring the incorporation [α-^{32}P]dCTP.

(d) Stop mix for nick-translation reaction

This mixture was composed of 2%(w/v) SDS, 50 mM EDTA, 10mM Tris-HCl, pH7.5.

2.8. Buffers for Restriction Enzymes

(a) 10 x EcoRI buffer

0.5 M	Tris-HCl pH7.4
0.5 M	NaCl
0.1 M	$MgCl_2$ (BDH)
10 mM	Dithiothreitol (Sigma)
1 mg ml^{-1}	Bovine serum albumin (Sigma)

(b) 10 x BamHI buffer

0.2 M	Tris-HCl pH 7.0
1 M	NaCl
70 mM	$MgCl_2$
20 mM	2 Mercaptoethanol (BDH)
1 mg ml^{-1}	Bovine serum albumin

(c) 10 x Hinc II buffer

0.1 M	Tris-HCl pH7.4
0.6 M	NaCl
0.1 M	$MgCl_2$
10 mM	Dithiothreitol
1 mg ml^{-1}	Bovine Serum albumin

2.9. Mating procedures

Donor strains carrying tetracycline resistance were grown overnight (18 hours), at 37°C in N2TG broth. The recipient strain Streptococcus faecalis JH2-2 (Jacob, A. & Hobbs, S.J., 1974) was grown under the same conditions. One millilitre of each strain was then sub-cultured into 25 ml of fresh N2TG broth and grown at 37°C in an environmental shaker until an optical density of 0.3 to 0.4 measured at 600 nm was achieved. At this

stage the cultures were quickly mixed using an initial ratio of one donor (0.5 ml of a donor culture) to 10 recipients (4.5 ml of the recipient culture), to give a final volume of 5 ml. One millilitre of this mixture was filtered through a sterile membrane filter (Sartorius, 0.45 μm pore size, 13 mm), which was subsequently placed filtrate side away from the agar, on a DST agar plate and incubated for eighteen hour at 37^{o}C. Controls comprising donor and recipient cells alone were treated similarily in order to estimate the frequency of spontaneously appearing drug resistant mutants in the population. A pre-incubation viable count was also perfomed using the method of Miles and Misra (Miles, A.A. & Misra, S.S., 1938) to determine the total number of donors and recipients present and to check their ratios.

After incubation, the cells on the filter were suspended in 1 ml of N2TG broth and the mixture was plated on DST plates selective for transconjugants. A post-incubation viable count was performed using the same method as the pre-incubation viable count. The frequency of transfer for each strain was calculated by dividing the number of transconjugants per millilitre by the post-incubation viable count of the donors cells in the mating mixtures.

Introduction of the sex factor, an erythromycin resistance plasmid, pAMβ1, (from Streptococcus faecalis strain DS5 (Clewell, D.B. et al., 1974)) was achieved using the same mating procedures as above. The donor strain was SSA13 (erythromycin and fusidic acid resistant, made by crossing JH2-2 with DS5 and selecting for

colonies showing resistance to fusidic acid and erythromycin only). The recipient strains were the original tetracycline resistant strains (Table 4). In order to check the genotypes of the transconjugants after the introduction of the sex factor the transconjugants on the double antibiotic tetracycline and fusidic acid plates were replica plated on to DST plates with fusidic acid, rifampin, tetracycline and erythromycin, using toothpick transfer.

In the case where the transfer frequency of tetracycline resistance was low, a secondary transfer mating using the primary transconjugants as donors and JH2-18 strain, with chromosomal resistance to rifampin and streptomycin and requiring thymine was used as a recipient. The secondary transconjugants were screened via replica plating for the presence of various markers.

2.10." Clumping inducing assay"

The sex pheromone production or "clumping inducing assay" was carried out using the method of Dunny and colleagues (Dunny, G.M. et al., 1979). This method was as follows:-
The plasmid-less JH2-2 Streptococcus faecalis strain, a strain known to be a "producer strain" i.e. produces pheromones, was grown in 25 ml N2TG broth until just prior to entering stationary phase.Cells were then pelleted by centrifugation at 1500 r.p.m. (MSE Centaur 2) for five minutes at $4^{o}C$ and the supernatant was filtered through a Millipore filter (0.22μm pore size). The filtrate was then autoclaved for 20 minutes, in order to stabilize the " clumping inducing " agent and the filtrate was stored a $4^{o}C$ until required.

The quantitation of pheromone activity was achieved using a

microtitre system (Sterilin microtiter plates, 96 well) involving the mixing of cells harbouring putative conjugative plasmids, responder cells, with JH2-2 filtrate. The filtrates were added to "U" shaped microtitre plates and a two-fold serial dilution (50 μl) into fresh N2TG broth was made. Responder cells grown to early stationary phase were diluted to an A_{660} of 0.5 in fresh N2TG broth and added to each well in a volume of 50 μl. The plates were incubated at 37^{o}C on a shaker for 120 minutes. After this period the plates were examined for the presence of flocculent material at the bottom of the wells resulting from clumping of cells. The Streptococcus faecalis strain DS5, which harbours the conjugative plasmids pAMβ1 and pAMγ1, was used as a control since this strain exhibits self-clumping in the presence of JH2-2 filtrate.

2.11. Small scale plasmid isolation from streptococci

Two methods,variations on the alkaline extraction procedure were used for the screening of plasmid DNA. The first was a modification by Dr. C. Drainas and Sheila Unkles of an alkaline lysis procedure (Dunny, G. et al., 1981).The theoretical basis of this procedure is the denaturation of the linear, but not the closed circular DNA,occurs over a narrow range of pH,between 12.0 to 12.5,thus alkali lysis can be used to enrich for closed circular DNA(Birnboim,H.C. & Doly,J.1979).In addition this procedure is both rapid and simple.

One millilitre of an overnight culture of the parental donor strains of group D streptococci, resistant to tetracycline, and their tetracycline resistant transconjugants (resulting from the

mating of a parental donor strain with the JH2-2 strain) was grown in 24 ml of BYGT broth supplemented with 20mM threonine (to prevent the formation of cross-linkages in the bacterial cell wall) and tetracycline (to enhance the tetracycline resistance determinant) for three hours in an environmental shaker at 37°C(Dunny,G.et al.

The bacterial strains were harvested by centrifugation at 10,000 r.p.m. (Sorvall SS34 rotor in a Sorvall RC5C centrifuge) for 10 minutes, the supernatant discarded and the pellet washed in 5 ml of TES buffer. The washed pellet was then re-suspended and centrifuged again at 10,000 r.p.m. for 10 minutes. the supernatant was again discarded and the pellet re-suspended in 0.2 ml TE buffer, followed by the addition of 50 µl of lysozyme (40 mg ml^{-1}), to break down the bacterial cell walls. After incubation for 20 minutes at 37°C, 4.8 ml of lysis buffer (1%(w/v) SDS in TE buffer, pH 12.45) was added to achieve alkaline denaturation of the DNA and the mixture gently shaken for 1 minute. This step was followed by incubation again for 20 minutes at 37°C after which 0.8 ml of 2 M Tris, pH 7 was added (to neutralize the lysate) and the sample gently shaken for 1 minute. Next, 0.54 ml of 5 M NaCl was added to remove high molecular weight DNA. The sample was them shaken for a few seconds and incubated overnight at 0°C.

After incubation at 0°C each sample was centrifuged at 15,000 r.p.m. for 30 minutes (Sorvall rotor SS34) and the supernatant saved and transferred into a corex tube. A volume of 4.8 ml of phenol saturated with 3%(w/v) NaCl was added (to remove

single stranded DNA), the sample was inverted 5 times to mix the contents thoroughly and them centrifuged at 2000 r.p.m. (MSE Centaur 2 centrifuge) for 5 minutes. The upper phase was saved and the phenol extraction repeated. Following phenol extraction, the aqueous phase was then extracted with an equal volume chloroform: isoamyl alcohol (24:1 v/v). The aqueous layer was again saved and 3 M sodium acetate was added and the DNA was separated from degraded RNA by ethanol precipitation using 2 volumes of -20oC 95% ethanol. The corex tube containing the sample was then inverted five times and the sample was stored overnight at -20^{o}C.

The DNA was pelleted by centrifugation at 12,000 r.p.m. (Sorvall rotor SS34) for 30 minutes and the pellet was re-suspended in 50μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

The second method used for the isolation of plasmid DNA was also an alkaline lysis procedure. The technique used was a modification by Dr. H. Siegrist of the method of Kado and Liu (Kado, D.I. and Liu, S.T. 1981).

Strains were grown overnight at 37oC in 3 ml of BHI broth supplemented with 20 mM Threonine and 2%(w/v) glucose. The cultures were centrifuged at 3,000 r.p.m. (Sorvall rotor SS34) for 10 minutes. The pellet was suspended in 0.5 ml of 0.01 M Tris-HCl with 25%(w/v) sucrose, pH 8, and subsequently 0.5 ml of lysozyme (40 mg ml^{-1}) in 0.25 M Tris, pH 8 was added and the strains incubated for 30 minutes at 37^{o}C. After incubation, 0.25 ml of 0.25 M EDTA was added followed by 2 ml of lysis solution

(50 mM Tris, 3%(w/v) SDS adjusted to pH 12.6). The sample was shaken gently until the lysate cleared and the DNA denatured by heating to 75°C for 30 minutes. After incubation, 2 volumes of phenol were added to the samples whilst still hot and gently shaken. In order to facilitate the removal of the aqueous phase,the aqueous phase was increased by adding one millilitre of distilled water. The samples were then shaken and centrifuged for 15 minutes at 4,500 r.p.m. (Sorvall rotor SS34) at room temperature. After centrifugation the aqueous phase was removed and 1 volume of isopropanol was added.The sample gently mixed and stored at -20°C for two hours in order to precipitate the plasmid DNA. After precipitation the samples were spun at 17,000 r.p.m. (Sorvall rotor SS34) for 30 minutes and the pellet was resuspended in 60 µl of TES buffer, pH 8.

2.12. Gel Electrophoresis

Agarose gel electrophoresis of streptococcal plasmids was carried out on the samples using a horizontal gel apparatus (Bethesda Research Laboratories, model HO). The samples were prepared for electrophoresis by adding 20 µl of each sample to 2.0 µl of bromophenol blue marker dye, and applying a volume of 20 µl of this mixture to the appropriate well of a 0.6%(w/v) agarose gel (20 x 25 x 0.7 cm) containing the intercalating stain ethidium bromide. The samples were electrophoresed into the gel at 100 V for 30 minutes and then at 60 V for 12 to 15 hours. The electrophoresis was performed at room temperature in 1 x TBE buffer.

After electrophoresis the plasmid bands were visualized by fluorescence under U.V. light, and photographed with a Polaroid MP-4 camera using type 655 film through Wrattan No. 23 plus ultraviolet filters. Molecular weight estimates of the plasmid bands in the samples were based on 4 standards, of known molecular weight pRP4-K215 (34 Md), AJ50 (10.1 Md), pR300B (5.7 Md) pBR325 digested with the restriction enzyme Bam HI (5.4 Md)(Boliver,F.,1978) and pBR322 (2.8 Md). Using these molecular weight standards a standard curve of the distance that the plasmid bands travelled in the agarose gel versus the $\log_{10}$ of the molecular weight of the standards was constructed and used to determine the molecular weights of the plasmids in the sample strains.

2.13. Digestion of streptococcal plasmid DNA with Bam HI or Eco RI.

Plasmid DNA from parental streptococcal strains was electrophoreses through 1%(w/v) low melting point agarose (sea plaque agarose, FMC Corp.) 1 X TBE buffer at 80 V for 4 hours at $4^{o}C$. The DNA wad recovered from the gel using the method of Weislander (Weislander, L., 1979). This method was as follows:-

(1) The putative plasmid DNA bands were cut out of the gel after viewing under U.V. transilluminator. Each band excised was placed in a separate sterile bijoux bottle and 5 volumes of 20 mM Tris-HCl (pH 8); 1 mM EDTA was added to each.

(2) The contents of the bijoux bottle were heated at 65oC for 5 minutes to melt the gel.

(3) The gel was extracted with an equal volume of phenol saturated with 0.1 M Tris, pH 8, at room tempertaure. The aqueous phase was then reextraced with phenol/chloroform (1:1 v/v) and chloroform at room temperature. The aqueous phase was collected in a 15 ml corex tube.

(4) The DNA was then precipitated by making the salt concentration up to 200 mM with 5 M NaCl followed by the addition of 2 volumes of ethanol.

(5) The DNA was pelleted by centrifugation at 10,000 r.p.m. (Sorvall rotor SS34) at 4°C. The pellet was washed twice in 70%(v/v)ethanol and the DNA resuspended in 20 µl of TE buffer. Since the amount of DNA obtained for each band was very small. Running on an agarose gel followed by extraction from the gel was undertaken four times. Identified bands of individual plasmid DNA were then pooled, and the DNA ethanol precipitation and resuspended in a final volume of 20 µl of TE buffer containing 10 µg ml^{-1} RNAse A. The plasmids were then ready for digestion with each of the Eco RI and Bam HI.

Digestion of DNA with restriction enzymes was carried out as follows:-

Plasmid DNA (0.5 µg)	20 µl
10 x enzyme buffer	2 µl
Eco RI or Bam HI (4U $µl^{-1}$)	1 µl
	23µl

The reaction was incubated at 37°C for 1 hour. The restricted DNA was extracted once with phenol/chloroform (1:1 v/v) and once with chloroform before washing in 70%(v/v) ethanol. The precipitate was resuspended in 25 µl of TE buffer and 2.5 µl of Bromophenol blue marker dye added. The DNA was electrophoresed through a 1%(v/v) agarose gel containing ethidium bromide (0.5 µg ml^{-1}) in 1 x TBE buffer at 100 V and the plasmid bands viewed with a U.V. transilluminator.

2.14. Isolation of chromosomal DNA from streptococci.

Chromosomal DNA was extracted using essentially the same method as that described by Burdett (Burdett, V. et al., 1982a). In this method, cultures of streptococci were grown overnight in 50 ml of BHI broth supplemented with 20 mM DL-threonine, 6.5 mM cysteine hydrochloride (Sigma) and 20 µg ml^{-1} tetracycline. Bacterial cultures were decanted into 50 ml Oakridge tubes (Sorvall) and centrifuged at 5,000 r.p.m. (Sorvall rotor SS34) for 10 minutes. The supernatant was discarded and the bacterial pellet resuspended in 12.5 ml TE(10) buffer (10 mM Tris-HCl; 10mM EDTA (pH 8)). Bacterial cells were then treated with 50 mg of lysozyme for 3 hours at 37°C. Following this, the cells were centrifuged again at 5,000 r.p.m. for 10 minutes and resuspended in 4 ml of TE(10) buffer. The cells were lysed by the addition of 0.5 ml of 20%(w/v) SDS and the lysate was treated with 100 g ml^{-1} of Proteinase K for 12 hours at 37°C. Each lysate was extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) and the DNA was precipitated by adding two volumes of ice cold 95% ethanol, and leaving overnight at -20°C.

DNA pellets were centrifuged at 10,000 r.p.m. (Sorvall rotor SS34), the pellets washed twice with 70%(v/v) ethanol and the DNA dissolved in 10 ml of TE buffer.

In the case of both plasmid and chromosomal DNA, the concentration of DNA was assessed by measuring the optical density of each sample at 260 nm on a PYE Unican PU8600 UV/VIS spectrophotometer. The DNA was stored at $4^{o}C$ until required.

2.15. Recombinant plasmids used in this study.

Recombinant plasmids containing tet L or tet M were constructed by Vickers Burdett and colleagues (Burdett, V. et al., 1982a,b; Inamine, J. & Burdett, V., 1982) for use in hybridization studies .

The Escherichia coli strain SK1592 (a plasmid less strain) was transformed with the recombinant pVB. A15 carrying the tet L determinant. This hybrid plasmid was composed of the plasmid pMV158 (5.2 kb) (Burdett, V. et al., 1982) from Streptococcus agalactiae (Group B) (Burdett, V., 1980) and Escherichia coli plasmid pVH2124 (8.64 kb) (So, M. et al., 1975).

Strain SK1592 was also transformed separately with the recombinant plasmid pJ13. This recombinant plasmid which carries the tet M determinant was a composed of the 5 kb Hinc II fragment encoding tetracycline resistance derived from Streptococcus agalactiae strain B109 chromosome DNA (Burdett, V. et al., 1982) and Escherichia coli plasmid pACYC177 (3.45 kb) (Chang, A.C.Y. & Cohen, S.N., 1978).

2.16. Isolation of recombinant plasmids from Escherichia coli.

Plasmids from Escherichia coli strains containing the recombinant plasmids were isolated using a modification of the method described by Birnboim and Doly (Birnboim, H.C. & Doly, J., 1979).

Strains harbouring recombinant plasmids were grown overnight at 37°C in 25 ml of L-Broth containing 20 µg ml-1 of tetracycline. These overnight cultures were then added to 475 ml of L-Broth and allowed to grow for a further 24 hours. Bacterial cultures were then centrifuged (Sorvall RC5C centrifuge)in polypropylene bottles (Sorvall) at 5000 r.p.m. (Sorvall GSA rotor) for 10 minutes at 4°C. The supernatant was removed and each of the two bacterial pellets resuspended in 9ml of solution I (lysis buffer) before being pooled to give 18ml total volume. A volume of 2 ml of lysozyme (40mg ml^{-1}) dissolved in solution I was added and the contests of the bottle were mixed will before leaving at room temperature for 10 minutes.

Next, 40 ml of solution II (alkaline solution) made up fresh, was added and the mixture was shaken hard and left on ice for 5 minutes. A volume of 20 ml of solution III (Neutralization solution) was added, the mixture shaken and left for 10 minutes on ice. After this, 5 ml of distilled water was added. The mixture was then centrifuged at 8000 r.p.m. (Sorvall GSA rotor) for 10 minutes at 4°C. The supernatant was filtered by pouring it into a fresh 250 ml bottle through two layers of absorbent cotton gauze (Smith and Nephew, type 13 light). The solution was allowed to warm to room temperature before the additon of 0.6 volumes (i.e. 48 ml) of isopropanol (BDH). After mixing the

solutions were left to stand for 15 minutes at room temperature before centrifuging at 8500 r.p.m. (Sorvall GSA rotor) for 20 minutes at 15°C. The supernatant was poured off and the polypropylene bottle inverted to drain off excess liquid. The pellet was then dried in a vacuum desiccator.

Following drying, the pellet was resuspended in 8 ml of TE buffer (pH 8). Once resuspended, 8 g of Caesium chloride (Koch Light, Ltd.) was added and allowed to dissolve. After adding 640 µl of ethidium bromide (10 mg ml^{-1}). The solution was dispensed into Beckman Quick seal tubes (16 x 76 mm) and after balancing the tubes, the solution was centifuged in a Beckman L8-M Ultracentrifuge for 30 minutes at 45,000 r.p.m. (Beckman rotor 70.1.ti), 8°C, followed by centrifugation at 35,000 r.p.m. for 68 hours under the same conditions. Plasmid DNA was removed using a needle and was dispensed into Beckman Quick seal tubes (13 x 51 mm) and the remaining volume of the tube was filled using Caesium chloride dissolved in TE buffer (pH 8) (1g ml^{-1}) and after balancing the tubes were centrifuged at 50,000 r.p.m. (Beckman rotor, Vti 65.2) for 18 hours at 8°C. After centrifugation plasmid DNA was removed, extracted 3 times with an equal volume of butanol (BDH) to remove ethidium bromide, and placed in dialysis tubing (Visking size 2-18/32'' Medicell Ltd) and dialysed for 18 hours in one litre of TE containing 0.1 M NaCl.

Following dialysis, the DNA was extracted twice with an equal volume of phenol/Choroform/isoamyl alcohol (24:24:1 v/v). The supernant was removed (2 ml) and 100µl of 5M NaCl was added and mixed before adding two volumes (4 ml) of ice cold 95% Ethanol. The DNA was precipitated at -20°C for 10 minutes before

pelleting the DNA by centriguation at 8,000 rpm (Sorvall rotor SS34) for 10 minutes. The supernatant was removed and the pellet dried in the vacuum desiccator before being resuspened in 200µl of TE buffer containing 10µg ml^{-1} of RNAse A (Sigma). The concentration of DNA was measured at 260 nm using a spectrophotometer.

2.17. Digestion of chromosomal and plasmid DNA with the restriction enzyme Hinc II

The chromosome of Streptococcus faecalis contains approximately 2,000 kb whereas plasmids in this study range between 4 kb to approximately 50 kb. This size difference should be borne in mind when planning DNA hybridization studies since more chromosomal DNA needs to be bound to the nitrocellulose filter than plasmid DNA in order to detect sequence homology by hybridization using a a nick translated DNA probe. Thus 5µg of chromosomal DNA and 100 ng of plasmid DNA were digested with Hinc II.

(a) Digestion of chromosomal DNA

10µl	chromosomal DNA (0.5µg l^{-1})
2µl	10 x Hinc II buffer
2µl	Hinc II (1.5 Uµ l^{-1})
6µl	double distilled water
20µl	

The reaction was incubated overnight at 37°C. The following morning an additional 2 µl of enzyme was added and the reaction left at 37°C for an additional hour. The DNA was extracted with an equal volume of phenol-chloroform (1:1 v/v). The DNA in the aqueous layer was the precipitated by the additon of 0.5 µl of 5 M NaCl followed by 4 µl of ice cold 95%(v/v) Ethanol and storage at -70°C for 10 minutes. DNA was pelleted, washed twice at 70% Ethanol and resuspended in 20 µl TE buffer. Bromophenol blue dye 2 µl was added just prior to electrophoresis.

(b) Digestion of plasmid DNA

10 µl	plasmid DNA (10 ng l^{-1})
2 µl	10 x Hinc II buffer
1 µl	Hinc II (1.5 U μl^{-1})
7 µl	double distilled water
20 µl	

The reaction mix was incubated for 1 hour at 37°C. No additional enzyme was added after this period of time and the DNA was then treated in the same manner as the chromosomal DNA.

2.18 Gel Electrophoresis of plasmid and chromosomal DNA

Plasmid (100 ng) and chromosmal (5 µg) DNA were loaded onto separate 0.8%(w/v) agarose gels containing 5 µg ml^{-1} ethidium bromide. Digested and undigested DNA were loaded next to each other, along with negative controls consisting of chromosomal DNA from JH2-2 and plasmid pMV120 (carrying tet N determinant)

derived from a Streptococcus agalactiae (Group B strain) and positive controls pVB.A15 (tet L) and pJI3 (tet M). The markers used consisted of uncut bacteriophage DNA (31.5 Md, 48.5 kb) (Biolabs) and cut with Hind III (Biolabs). Digestion of λ with Hind III yields 8 fragments whose sizes are as follows:-

Fragment	Number of kb	Md
1	23.1	15
2	9.4	6.12
3	6.6	4.26
4	4.37	2.84
5	2.32	1.51
6	2.03	1.32
7	0.56	0.37
8	0.13	0.08

The gel was run at 80 V for 4 hours in 1 X TBE buffer. The DNA was then examined using a U.V. transilluminator.

2.19 Blotting of DNA onto nitrocellulose filters

Treatment of DNA prior to blotting and the blotting of DNA onto nitrocellulose was carried out according to the methods of Southern and Sealey (Southern, E. 1975; Sealey, P. and Southern, E. 1982) with modifications by Wahl (Wahl, G.M. et al., 1979)

(A) Treatment of DNA prior to blotting

The chromosomal DNA isolated from streptococci has a size greater than 20 kb and since these large molecules do not transfer efficiently DNA was nicked using a 254 nm U.V. lamp. prior to transferring the DNA form the gel to the nitrocellulose filter, the DNA was denatured by immersing it in alkali followed by neutralization of the gel. This procedure was as follows.

(1) The gel was placed in a box and immersed is 1.5M Nacl, 0.5 M NaOH and gently agitated for 1 hour.

(2) The gel was then washed with distilled water.

(3) The gel was washed in neutralizing buffer, 1.5M NaCl, 1.0M Tris (pH 8) with gentle agitation for 1 hour.

The denaturation results in the production of single stranded DNA suitable for hybridizing to nick translated probe

(B) Treatment of Nitrocellulose filters prior to blotting

A piece of nitrocellulose paper (BA85, Schleicher and Schuell GmBH) sufficient to cover the area of the agarose gel was cut out and soaked for 30 minutes in distilled water to wet it followed by soaking in 2 x SSC for 30 minutes before use in the DNA transfer.

(C) Setting up the DNA transfer system

The DNA transfer system was set up as shown in Figure 3

The DNA was left overnight to transfer onto nitrocellulose filter

(D) Treatment Of the agarose gel and Nitrocellulose filters

Following overnight transfer the completeness of DNA transfer was checked by immersing the agarose gel in 1 x TBE buffer containing 0.5 μg ml^{-1} of ethidium bromide for 30 minutes followed by destaining in 1 x TBE for 30 minutes. the gel was then viewed on a U.V. transilluminator box to detect the presence of DNA.

The nitrocellulose filters were treated as follows:-

The filters were allowed to air dry and they were then placed between Whatman 3 mm paper and baked in a vacuum oven at 80^{o}C for 2 hours. The filters were then used in the hybridization studies.

2.20. Nick - Translation of probes and DNA:DNA hybridization

Tet L and tet M probes were nick translated using the Amersham nick translation kit (N.5000). The protocol used for labelling was a modification by A. Ford of that described in the Amersham nick translation kit instuction booklet. The protocol was as follows:-

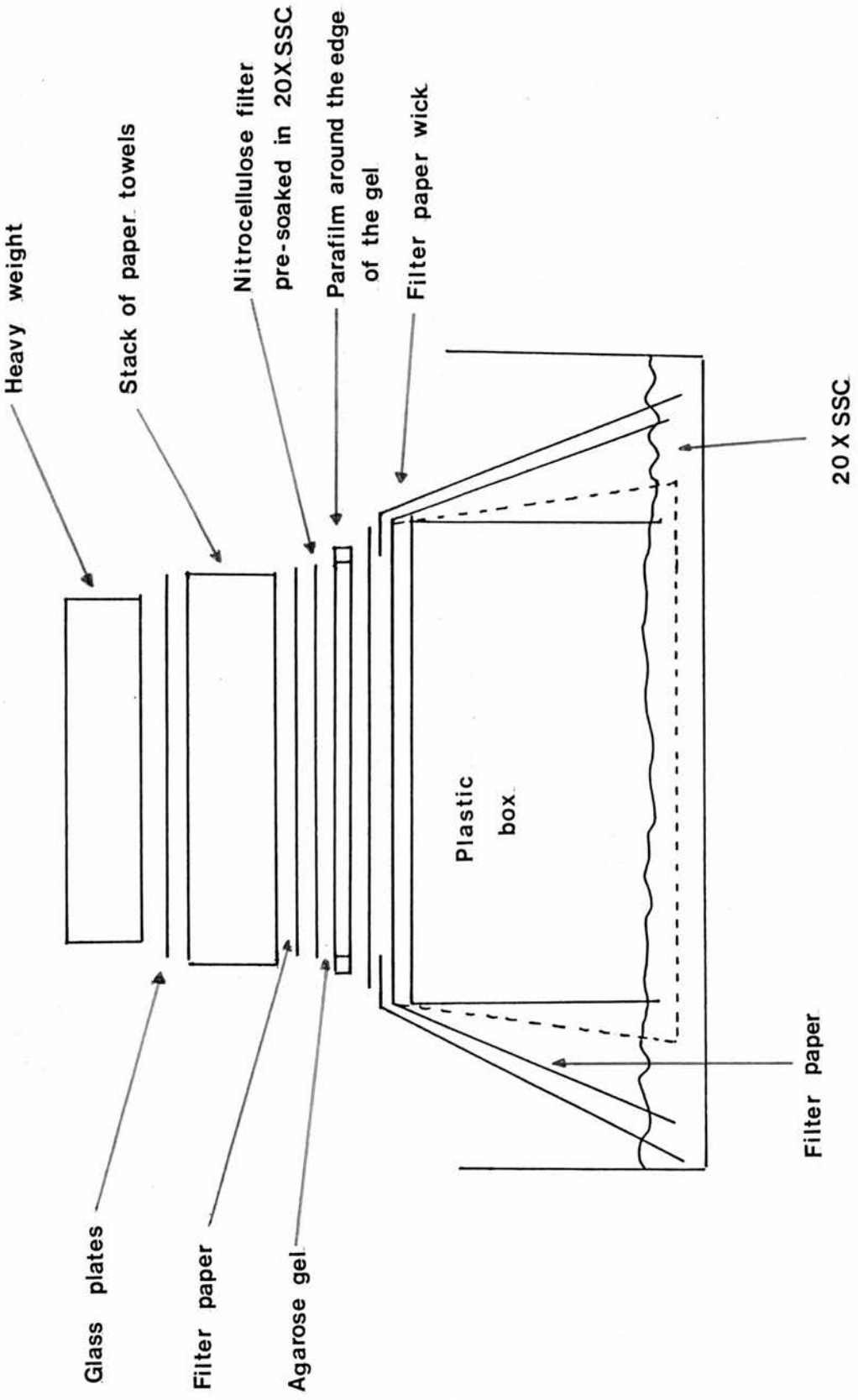


Figure 3 Transfer of DNA from an agarose gel to a nitrocellulose filter

1) A volume of 30μl of α-^{32}P dCTP (1m Ci ml^{-1}; specific activity 410 Ci mmol^{-}1, 37MBq ml^{-1}) (PB165 Amersham) was placed in a 1.5 ml microcentrifuge tube and the radioisotope was dried down in a vacuum dessictor

2) The radioisotope was resuspended in 10μl of distilled water by mixing. To this was added in order:- One microlitre of DNA (dissolved in distilled water to give a final concentration of 100 ng l^{-1}), 4μl of Amersham kit solution 1 and 3μl of distilled water. The contents of the tube were gently mixed and 2 1 of Amersham kit solution 2 was added. The contents of the tube were again gently mixed and the reaction mix was incubated at 15^{o}C for 60 minutes.

3) After 60 minutes, 20μl of 'stop mix' was added to reaction mixture and the contents of the microcentrifuge tube were mixed.

4) Following mixing, 4μl off 3 M potassium acetate (pH 5.6), 40 μl of Amersham kit solution 3 and 30μl of ice cold 95%(v/v)ethanol were added. After the additon of each reagent the contents of the tube were mixed. The reaction mixes were then placed at -20^{o}C for 10 minutes to enable the DNA to precipitate.

5) The DNA was then pelleted by centrifuging at 11,000 rpm (M.S.E. Micro Centaur) for 10 minutes, the supernatant removed and the pellet washed in 70% ethanol.

6) The DNA pellet was resuspended in 200μl of 10 mM Tris, pH 8.

Following this the DNA was again precipitated using 20 μl of 3 M potassium acetate (pH 5.6) and 500 μl of ice cold 95% ethanol.

7) The DNA was again pelleted by centrifuging at 11,000 r.p.m. for 10 minutes, the pellet washed in 70% ethanol and the DNA resuspended in 500 μl of 10 mM Tris, pH 8.

The amount of radioisotope incorported into the probe was estimated by removing 3 μl of each reaction mix and spotting it onto a GF/C filter (2.5 mm, Whatman). Once the filter was dried it was placed in 5 ml of scintillant (Emulsifier Scintillator 199, United Technologies Packard) and counted on a LKB 1217 Rackbeta liquid scintillation counter. DNA probes with specific activities greater than 10^8 cpm/μg were used in the hybridization studies.

(a) Preparation of the nitrocellulose filter prior to hybridization to the nick translated probe.

The baked nitrocellulose filter was placed in a heavy plastic bag and 15 ml of 3 x SSC was added to the bag before sealing after air bubbles had been removed. The filter was then incubated at 68^{o}C for 1 hour in a shaking incubator. This step dilutes salts present on the filter which would affect hybridization. After an hour the bag was opened the 3 x SSC poured out and replaced by 15 ml of prehybridization buffer, the bag resealed and the filter incubated for a further hour at 68^{o}C in a shaking incubator. The the prehybridization buffer was discarded after an hour and replaced by hybridization buffer. The

bag was sealed one more time and the filter incubated at 68°C for 30 minutes in a shaking incubator.

(b) Preparation of the nick translated probes prior to hybridization.

Immediately prior to hybridization, nick translated probes were boiled for 5 minutes at 100°C to seperate the two DNA strands. The probes were then cooled on ice to prevent the strands from reannealing. Plastic bags containing nitrocellulose filters incubated for 30 minutes in hybridization buffer at 68°C were opened and the appropriate radiolabelled probe added. Air bubbles were removed the bags resealed and incubated overnight at 68°C in a shaking incubator.

(c) Washing of nitrocellulose filters and autoradiography.

After overnight incubation the hybridization buffer containing the probe was discarded and the nitrocellulose filter removed from the plastic bag. The filter was washed 3 times at 68°C in "blot" washing solution. At the end of each wash the filter was monitored with a Geiger counter to assess the background level of α-^{32}P dCTP.

The stringency conditions used for the washing of the nitrocellulose filters,subsequent to their hybridization with the radiolabelled probes,was calculated using relationships based on various DNA parameters(Dove,W.F. & Davidson,N.,1962;Marmur,J. & Doty,P.,1962;Bonner,T. et al.,1973;Maniatis,T. et al.,1982).The conditions used assumed that the probe had a 50% guanine plus

cytosine content and a DNA melting temperature of 80oC,thus,the washing of the nitrocellulose filters was carried out at 68oC using 0.1 X SSC.These conditions are quite stringent,therefore an 80 to 95% match between the DNA bound to the nitrocellulose filter and the probe is required in order to detect homology via hybridization.

The nitrocellulose filter was then air dried and the dried filter expossed to Kodak X-Omat XAR-5 film plus intensifying screens at $-70^{o}C$.

2.21. Bacteriocin production.

The strains were tested for bacteriocin production using a modification by Dr. A. Jacob of method of Franke and Clewell

(Franke, A.E. & Clewell, D.B., 1980).

Test strains were streaked onto BHI agar plates and grown overnight. A bacteriocin producer strain of Streptococcus faecalis JH1-23, resistant to fusidic acid and containing the hemolysin-bacteriocin producing plasmid, pJH2, was included on the plate along with a known non-producer strain, a plasmid-free antibiotic sensitive strain, JH1-18. After incubation, one colony was picked from a plate containing the indicator strain, JH2 (bacteriocin sensitive) this colony was inoculated into 0.5 ml BHI broth and was vortexed. A 0.5 ml aliquot of BHI broth containing 1%(w/v) agar was then mixed with the innoculated colony, and 1 to 2 drops of this mixture were pipetted onto the test strains, and incubated for 5 hours.

2.22. Curing with Novobiocin.

The method used was a modification by Dr. H. Siegrist of the technique used by McHugh and Schwartz (McHugh, G. and Schwartz, M., 1977).The ten parental strains containing a single plasmid (see table 8) were chosen for curing studies because elimination of plasmid DNA concomitant with the loss of tetracycline resistance would indicate that the plasmid DNA present in each of these strains carried a tetracycline resistance determinant.Novobiocin was selected as the curing agent because of its reported high efficiency of curing plasmid DNA in Streptococcus faecalis(Jacob,A.E. & Hobbs,S.,1974;Jacob,A.E.personal communication).

Strains to be cured were grown overnight in 10ml of BHI

broth at 37oC.These cultures were then diluted in BHI broth to give a concentration of 10^5 cells ml-1.two millilitres were then grown in a range of doubling concentrations of novobiocin ranging from 0µg ml-1 to 125µg ml-1 in order to determine the minimum inhibitory concentration (M.I.C.) of the drug.After 18 hours incubation at 37oC,0.1 ml of cultures containing a sub-inhibitory concentration of novobiocin (8µg ml-1 in the cases where the M.I.C. was 16µg ml-1 and 16µg ml-1 where the M.I.C. was 32µg ml-1),were plated out at a concentration of 10^4 cells ml-1.Streptococcus faecalis strain JH1-2 was included as a positive control.This strain contains the plasmid pJH1 encoding resistance to erythromycin,kanamycin,streptomycin and tetracycline and has a high efficiency of curing of approximately 25%(Jacob,A.E.,personal communication)when treated with 8µg ml-1 novobiocin.Negative controls,cultures containing no novobiocin,were also included.Both treated and untreated controls were also plated out at 10^4 cells ml-1.

The plates were then incubated at 37°C overnight. A random sample of 500 colonies were picked using toothpicks from each novobiocin treated strain and their corresponding controls and these colonies were patched onto BHI agar master plates. After an overnight incubation colonies from parental and transconjugant strains were replica plated using the velvet method, onto single antibiotic BHI agar plates containing 20µg ml^{-1} tetracycline, and onto BHI agar plates with no antibiotic. Transconjugant strains were also replica plated onto single antibiotic BHI agar plates containing 100µg ml^{-1} rifampin and 25µg ml^{-1} fusidic acid. A random sample of 10 colonies from each strain were examined for plasmid content.

2.23. Storage of Parental Strains and their transconjugants.

The parental strains and transconjugants were inoculated into small vials containing 1.5 ml of Luria broth with 20μg ml^{-1} of tetracycline and incubated overnight at 37^{o}C. Following incubation 1.5 ml of filter sterlized 10% glycerol/10% DMSO (v/v) was added to each vial and the strains stored at -70^{o}C.

3. RESULTS

3. RESULTS

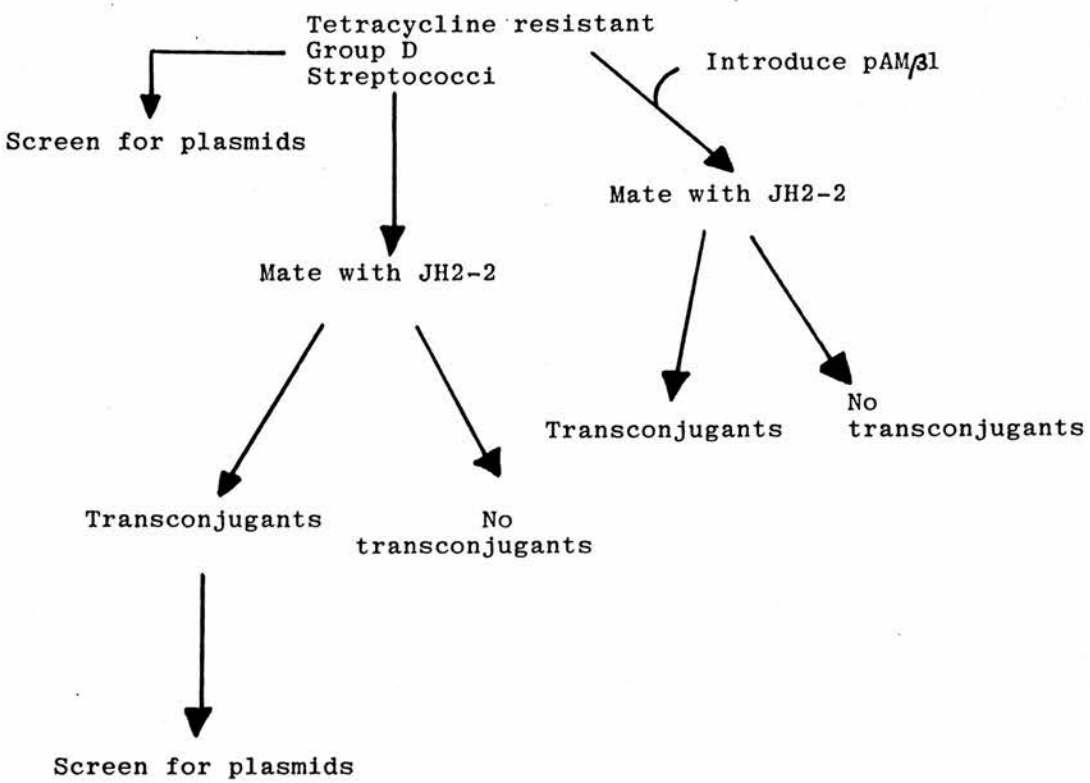
The regime used to screen tetracycline resistant strains for plasmid content is shown in Figure 4.

3.1. Transferability of the tetracycline resistance determinant in the absence of the conjugative plasmid pAMβ1.

The recipient strain used in the first series of 18 hour matings was the JH2-2 strain of Streptococcus faecalis which contains no plasmids and carries chromosomal resistance markers for fusidic acid and rifampin. Fusidic acid was used as a selective marker along with tetracycline in the selection of transconjugants. Rifampin was used as the non-selective marker. The frequency of mutation to a high level of resistance to rifampin and fusidic acid is extremely low among the Group D streptococci and spontaneous mutation to tetracycline occurred at very low levels of less than 10^{-10}(Franke,A. & Clewell,D.B.,1981).

Out of the 30 strains (Table 5) none of the strains showed a remarkably high frequency of tetracycline resistance (i.e. 10^{-4} to 10^{-1} per donor). Twenty-three strains transferred tetracycline resistance to JH2-2. The frequency of transfer ranged between 2.7×10^{-5} by 2.2×10^{-9} transconjugants per donor. The majority of strains transferred their tetracyline resistance in the range of 1×10^{-6} to 1×10^{-8}. Seven strains produced no transconjugants. These seven strains were all sewage isolates. The other four sewage isolates transferred their

FIGURE 4.



Scheme for the identification of tetracycline resistance plasmids.

TABLE 5

Transfer of tetracycline resistance with JH2-2 as recipient using the filter mating technique.

MEAN and STANDARD ERROR		
DONOR	WITHOUT pAMβ1	WITH pAMβ1
K4	$2.7 \times 10^{-5} \pm 1.27 \times 10^{-5}$	$5.8 \times 10^{-5} \pm 3.44 \times 10^{-5}$
K22	$1.7 \times 10^{-6} \pm 9.60 \times 10^{-7}$	$7.4 \times 10^{-8} \pm 3.02 \times 10^{-9}$
K37	$1.5 \times 10^{-6} \pm 5.36 \times 10^{-7}$	$7.9 \times 10^{-7} \pm 5.31 \times 10^{-8}$
K42	$8.0 \times 10^{-6} \pm 3.24 \times 10^{-6}$	$2.2 \times 10^{-6} \pm 1.67 \times 10^{-6}$
K45	$1.1 \times 10^{-8} \pm 1.00 \times 10^{-9}$	$2.0 \times 10^{-6} \pm 1.62 \times 10^{-6}$
K47	$1.5 \times 10^{-7} \pm 5.53 \times 10^{-8}$	$7.3 \times 10^{-8} \pm 5.20 \times 10^{-8}$
K48	$4.3 \times 10^{-7} \pm 1.53 \times 10^{-7}$	$7.6 \times 10^{-7} \pm 2.81 \times 10^{-7}$
K52	$1.2 \times 10^{-6} \pm 1.11 \times 10^{-7}$	$2.6 \times 10^{-6} \pm 2.36 \times 10^{-6}$
K54	$1.8 \times 10^{-7} \pm 6.17 \times 10^{-8}$	$2.1 \times 10^{-6} \pm 2.30 \times 10^{-7}$
K57	$3.7 \times 10^{-9} \pm 1.36 \times 10^{-9}$	$1.1 \times 10^{-7} \pm 1.75 \times 10^{-8}$
K58	$2,2 \times 10^{-9} \pm 1.32 \times 10^{-10}$	0
K59	$1.0 \times 10^{-8} \pm 2.64 \times 10^{-9}$	$1.1 \times 10^{-6} \pm 4.22 \times 10^{-7}$
K65	$1.5 \times 10^{-8} \pm 4.06 \times 10^{-9}$	$1.6 \times 10^{-7} \pm 7.25 \times 10^{-8}$
K76	$7.1 \times 10^{-6} \pm 2.13 \times 10^{-6}$	0
K78	$1.6 \times 10^{-8} \pm 5.82 \times 10^{-9}$	$1.1 \times 10^{-7} \pm 3.92 \times 10^{-8}$
K89	$1.5 \times 10^{-6} \pm 9.27 \times 10^{-7}$	ND
K91	$3.5 \times 10^{-8} \pm 1.91 \times 10^{-8}$	ND
K93	$4.5 \times 10^{-8} \pm 2.33 \times 10^{-8}$	0
K97	$1.8 \times 10^{-6} \pm 1.62 \times 10^{-6}$	$1.8 \times 10^{-6} \pm 1.52 \times 10^{-7}$
SB38	$2.0 \times 10^{-6} \pm 1.04 \times 10^{-7}$	0
SB62	$1.5 \times 10^{-7} \pm 1.01 \times 10^{-7}$	$2.7 \times 10^{-8} \pm 6.14 \times 10^{-9}$

TABLE 5 cont.

Transfer of tetracycline resistance with JH2-2 as recipient using the filter mating technique.

MEAN and STANDARD ERROR		
DONOR	WITHOUT pAMβ1	WITH pAMβ1
SB65	0	$2.5 \times 10^{-5} \pm 1.10 \times 10^{-6}$
SB68	0	$5.8 \times 10^{-8} \pm 1.25 \times 10^{-8}$
SB74	$1.4 \times 10^{-7} \pm 1.22 \times 10^{-8}$	$4.7 \times 10^{-8} \pm 1.82 \times 10^{-8}$
SB77	0	$6.2 \times 10^{-8} \pm 3.34 \times 10^{-8}$
SB79	0	$3.6 \times 10^{-8} \pm 1.10 \times 10^{-8}$
SB80	$1.6 \times 10^{-8} \pm 2.02 \times 10^{-9}$	$8.0 \times 10^{-8} \pm 4.41 \times 10^{-9}$
SB88	0	$4.5 \times 10^{-7} \pm 1.82 \times 10^{-8}$
SA13	0	0
SA92	0	$3.8 \times 10^{-8} \pm 1.63 \times 10^{-9}$

These transfer frequencies per donor give the approximate range, and mean, of at least four mating experiments.

ND = Not Determined.

resistance at a frequency between 2.07×10^{-6} and 1.6×10^{-8} per donor, these frequencies of transfer being on a par with those of the clinical isolates.

As mentioned previously, the frequency of spontaneous mutation to fusidic acid, rifampin and tetracycline amongst Group D streptococci is very low, thus it was assumed that transconjugants showing resistance markers for tetracycline resistance, fusidic acid resistance and rifampin were "true"

transconjugants and not just spontaneuos mutations.

Presumptive transconjugants from the first series of matings were selected on agar plates containing both tetracycline (20 μg^{-1}) and fusidic acid (25μg ml^{-1}). Transconjugants were picked and patched using toothpicks onto DST agar master plates and after overnight incubation at 37^{o}C these transconjugants were replica plated, using the velvet method onto DST agar plates and single antibiotic plates containing tetracycline (20μg ml^{-1}) fusidic acid (25μg ml^{-1}) and rifampin (100μg ml^{-1}). All transconjugants selected showed resistance to all three antibiotics.

3.2. Re-transfer of tetracycline resistance determinants in the cases of low frequency transfer strains.

Nine parental strains showed a frequency of transfer between 10^{-8} and 10^{-9} per donor which is approaching the spontaneous mutation frequency of Group D streptococci to tetracycline. The nine transconjugant strains were therefore examined to ascertain whether they were able to re-transfer their tetracycline resistance. These nine primary transconjugant strains LA5, LA10, LA11, LA12, LA13, LA15, LA17, LA18 and LA27 were subjected to a second series of 18 hour matings using the thymine requiring strain JH2-18 (which expresses chromosomal resistance to streptomycin and rifampin) as the recipient strain and the nine tetracycline resistant tranconjugant strains as donors. The transconjugants produced from the mating of the primary transconjugants with strain JH2-18 were defined as secondary

transconjugants. These secondary transconjugants were selected on BHI agar plates containing thymine ($2\mu g ml^{-1}$) tetracycline ($20\mu g$ ml^{-1}) and streptomycin ($2000\mu g\ ml^{-1}$). After picking and patching onto thymine containg BHI agar masterplates followed by overnight incubation at $37^{o}C$, the colonies were replica plated, using the velvet method, onto single antibiotic thymine BHI plates containing streptomycin ($2000\mu g\ ml^{-1}$), tetracycline ($20\mu g\ ml^{-1}$), rifampin ($100\mu g\ ml^{-1}$) and plates containing no antibiotic. Each of the transconjugants tested showed resitance to all four antibiotics.

All nine of the primary transconjugants transferred their tetracyline resistance and the frequency of transfer was again very low (Table 6) but comparable to those frequencies obtained in the first series of matings with JH2-2.

3.3. Transferability of the tetracycline resistance determinant in the presence of the conjugative plasmid pAMβ1.

The conjugative plasmid pAMβ1, a 16 Md plasmid carrying the erythromycin resistance determinant was introduced into the

TABLE 6

Mating of primary transconjugants with a JH2-18 strain expressing chromosomal resistance to streptomycin, rifampin and requiring thymine for growth.

STRAIN	TRANSFER FREQUENCY PER TETRACYCLINE RESISTANT DONOR
LA5(K45 x JH2-2)	$2.6 \times 10^{-8} \pm 0.14 \times 10^{-8}$
LA10(K57 x JH2-2)	$5.7 \times 10^{-9} \pm 0.81 \times 10^{-9}$
LA11(K58 x JH2-2)	$3.4 \times 10^{-8} \pm 0.73 \times 10^{-8}$
LA12(K59 x JH2-2)	$3.9 \times 10^{-8} \pm 0.12 \times 10^{-8}$
LA13(K65 x JH2-2)	$1.3 \times 10^{-8} \pm 0.36 \times 10^{-8}$
LA15(K78 x JH2-2)	$2.7 \times 10^{-8} \pm 0.22 \times 10^{-9}$
LA17(K91 x JH2-2)	$4.1 \times 10^{-8} \pm 0.27 \times 10^{-8}$
LA18(K93 x JH2-2)	$5.6 \times 10^{-8} \pm 0.81 \times 10^{-8}$
LA27(SB80 x JH2-2)	$4.6 \times 10^{-9} \pm 0.92 \times 10^{-9}$

These transfer frequencies per donor give the approximate range and mean of at lease 4 mating experiments.

tetracycline resistant parental strains by mating them with the erythromycin donor strain SSA13. The erythromycin resistance determinant was transferred at a frequency of 10^{-3} to 10^{-1} per donor. The resultant tetracycline and erythromycin resistant strains were then mated with the plasmid-free strain JH2-2 to determine whether or not the sex factor pAMβ1 would increase the frequence of transfer of the tetracycline resistance determinant.

Analysis of the transfer of erythromycin resistance and tetracycline resistance, revealed that these two resistances were not transferred at the same frequency,indicating that the two resistances were not carried by the same genetic element.The erythromycin resistance was transferred at a frequency between 8 x 10^{-1} to 9 x 10^{-5} per donor where as tetracycline resistance was transferred at a frequency between 5.8 x 10^{-5} to 2.7 x 10^{-8} per donor.

The tetracycline resistance determinant was mobilised in 9 strains; K45, K57, K59, SB65, SB68, SB77, SB79, SB88 and SB92. These strains exhibited an increase in the frequency of transfer of tetracycline resistance of 100 fold or greater. Four strains K58, K76, K93 and SB38 that had previously shown a transfer of their tetracycline resistance failed to do so in the presence of pAMβ1. In fact, K76 decreased its frequency of transfer from 7.3 x 10^{-6} per donor to 0. Six of the seven sewage isolates that had failed to transfer tetracycline resistance in the absence of pAMβ1 exhibited mobilization of their tetracycline resistance determinant in the presence of the sex factor. In the case SB65,

a frequency of transfer of 2.5×10^{-5} per donor was demonstrated. This frequency was in the same order as that of K4, the strain exhibiting the highest transfer frequency (2.7×10^{-5} per donor), in the absence of pAMβ1.

3.4." Clumping inducing agent" assay.

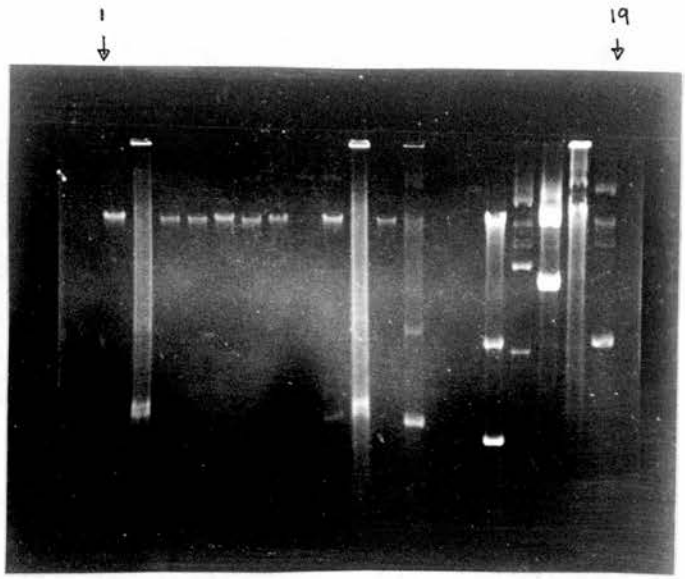
Pheromone production is associated with strains of bacteria that contain large conjugative plasmids and consequently this enables them to transfer their markers at a high frequency 10^{-3} to 10^{-1} transconjugants per donor. The large plasmids detected in the strains in the study did not appear to be conjugative but it was of interest to confirm whether or not the strains carrying plasmids could be induced to self clump when mixed with JH2-2 filtrate. As a positive control for these experiments, a known responder strain, Streptococcus faecalis DS5 which harbours pAMα1 (a non-conjugative plasmid which carries tetracycline resistance), pAMγ1, pAMγ2, pAMγ3 (which encode hemolysin and bacteriocin) and pAMβ1 (a conjugative plasmid encoding erythromycin resistance)(Clewell,D.B.,1981a) was used. Strain DS5 was shown to be induced to clump by a filtrate of strain J52-2 that had been diluted up to 32 fold. None of the 30 parental strains used in this study showed any response to the filtrate.

3.5. Isolation of plasmids and estimation of their molecular weights.

Lysates of the parental strains and transconjugants were

screened for plasmid DNA using two alkaline lysis procedures followed agarose gel electrophoresis (Figures 5 to 12). Both screening methods gave the same results.

FIGURE 5 - Analysis of Plasmid DNA from Parental and Transconjugant Strains using Gel Electrophoresis (Strains LA7 to K4)



Lane Number	Strain
1	LA7
2	K48
3	LA6
4	K47
5	LA5
6	K45
7	LA4
8	K42
9	LA3
10	K37
11	LA2
12	K22
13	LA1
14	K4
15	pBR322
16	pBR325/Bam HI
17	pAJ50
18	pRP4-K215
19	DS5

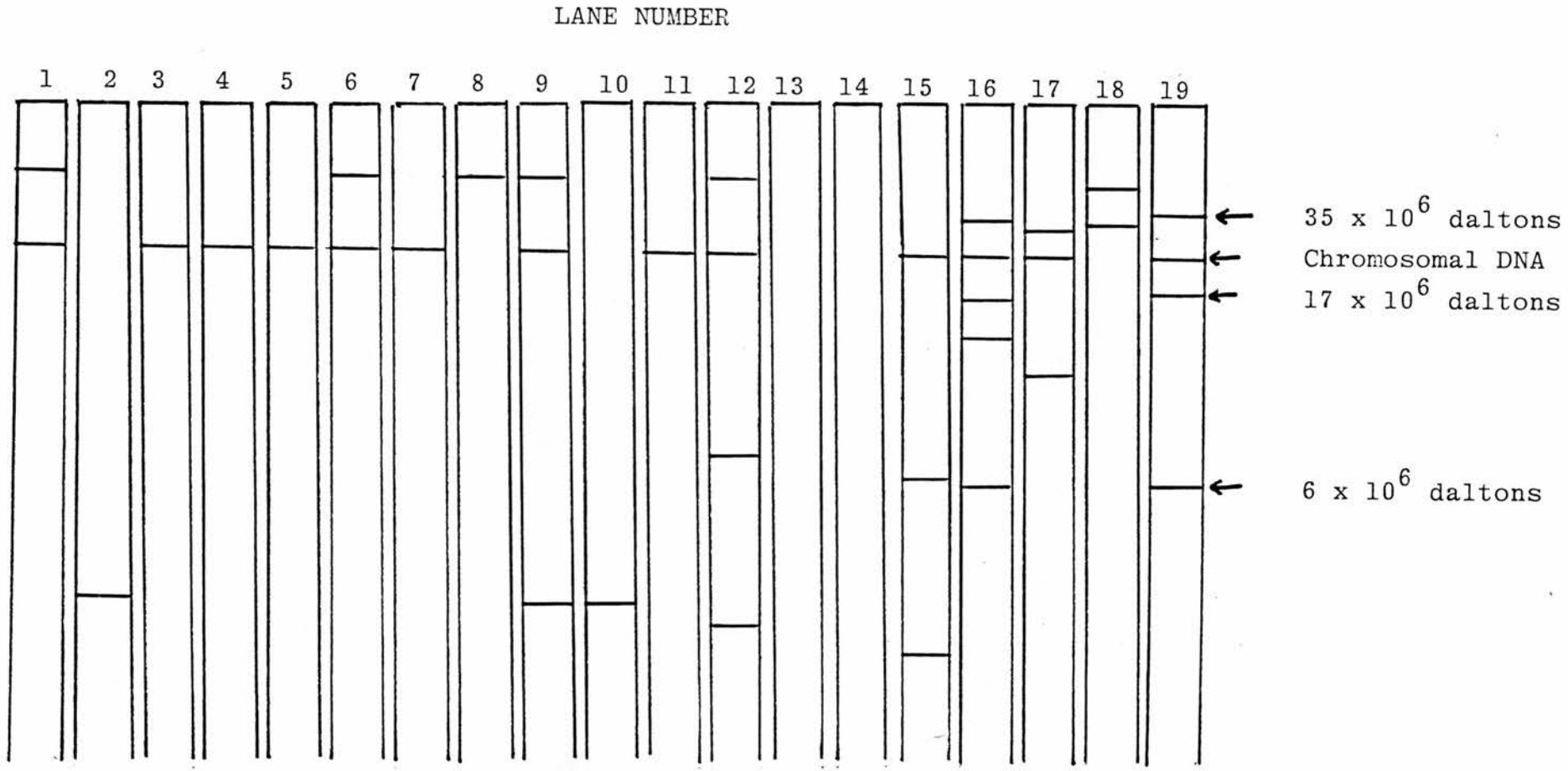
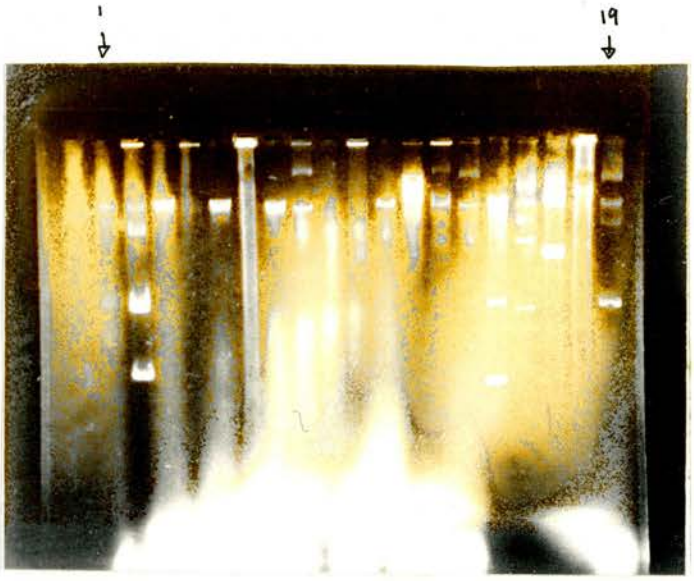


FIGURE 6. Schematic representation of Figure 5.

FIGURE 7 - Analysis of Plasmid DNA from Parental and Transconjugant Strains Using Gel Electrophoresis (Strains LA14 to K52)



Lane number	Strain
1	LA14
2	K76
3	LA13
4	K65
5	LA12
6	K59
7	LA11
8	K58
9	LA10
10	K57
11	LA9
12	K54
13	LA8
14	K52
15	pBR322
16	pBR325/Bam HI
17	pAJ50
18	RP4-K215
19	DS5

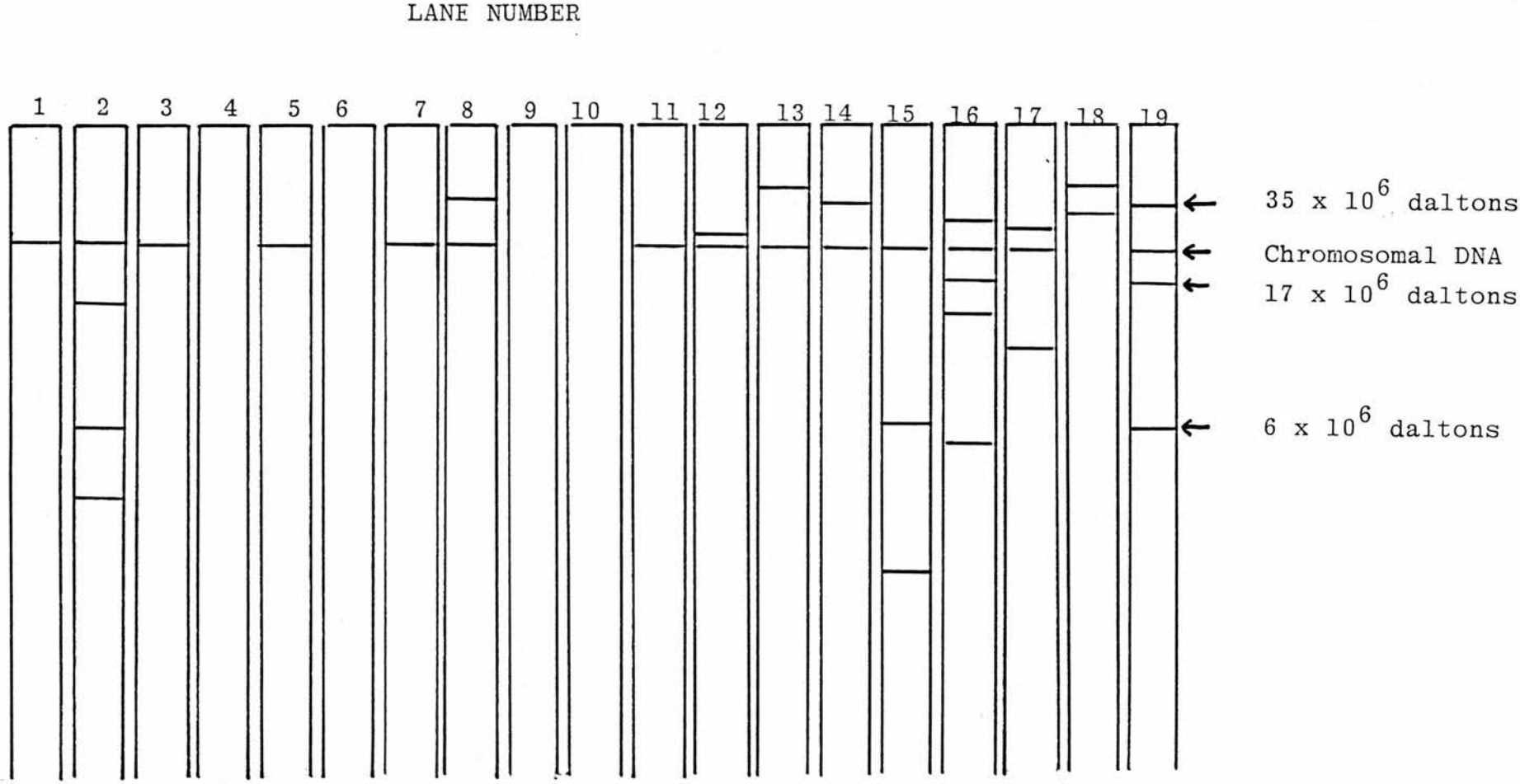
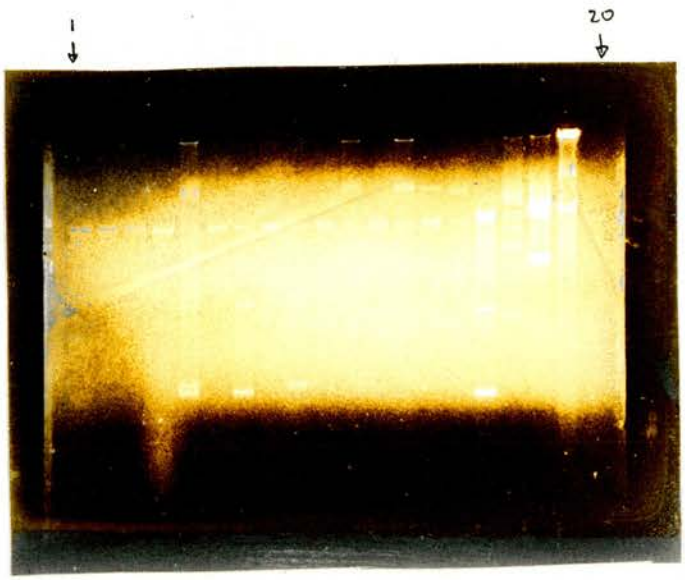


FIGURE 8. Schematic representation of Figure 7.

FIGURE 9 - Analysis of Plasmid DNA from Parental and Transconjugant Strains using gel electrophoresis (Strains SB68 to K78)



Lane Number	Strain
1	SB65
2	LA21
3	SB62
4	LA20
5	SB38
6	LA19
7	K97
8	LA18
9	K93
10	LA17
11	K91
12	LA16
13	K89
14	LA15
15	K78
16	pBR322
17	pBR325/ Bam HI
18	pAJ50
19	RP4-K215
20	DS5

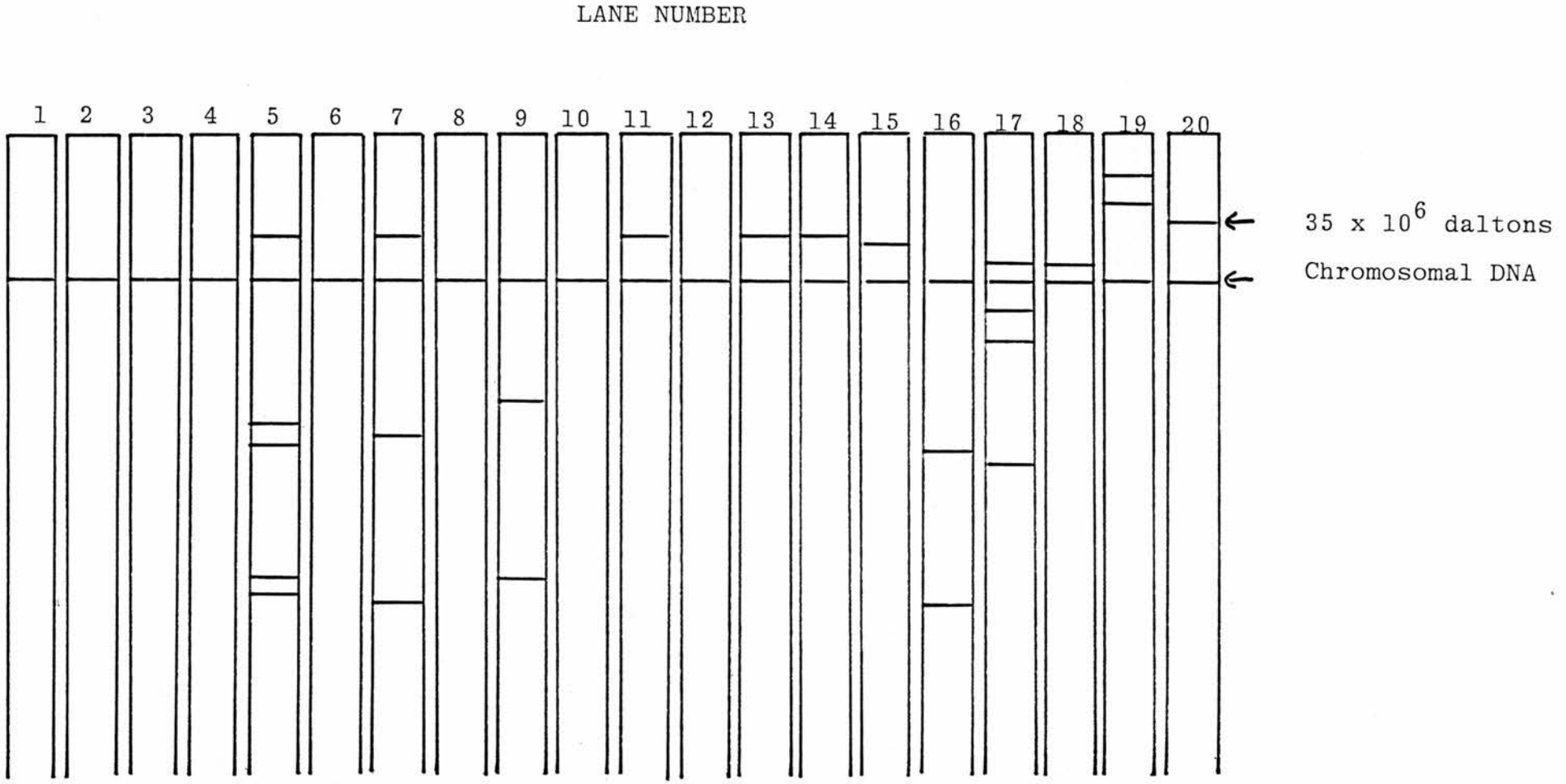
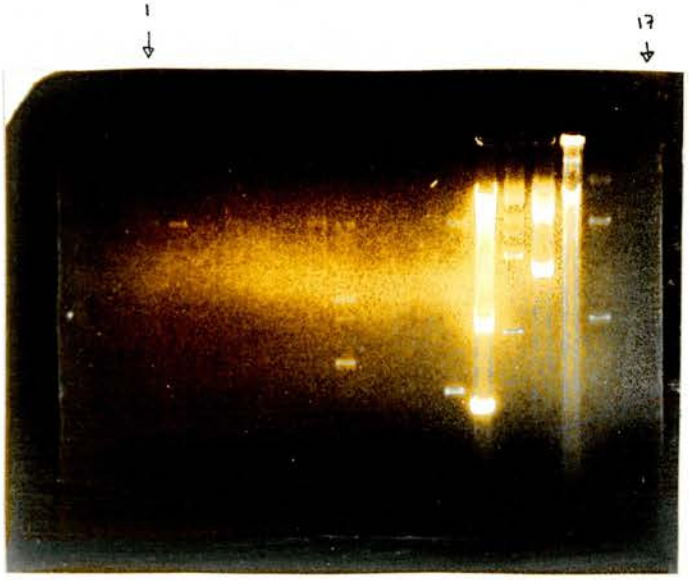


FIGURE 10. Schematic representation of Figure 9.

FIGURE 11. - Analysis of Plasmid DNA from Parental and Transconjugant Strains using Gel Electrophoresis (Strains SA92 to SB68)



Lane Number	Strain
1	SA92
2	SA13
3	SB88
4	LA27
5	SB80
6	LA26
7	SB79
8	SB77
9	
10	LA24
11	SB74
12	SB68
13	pBR322
14	pBR325/Bam HI
15	pRP4-K215
16	DS5
17	JH2-2

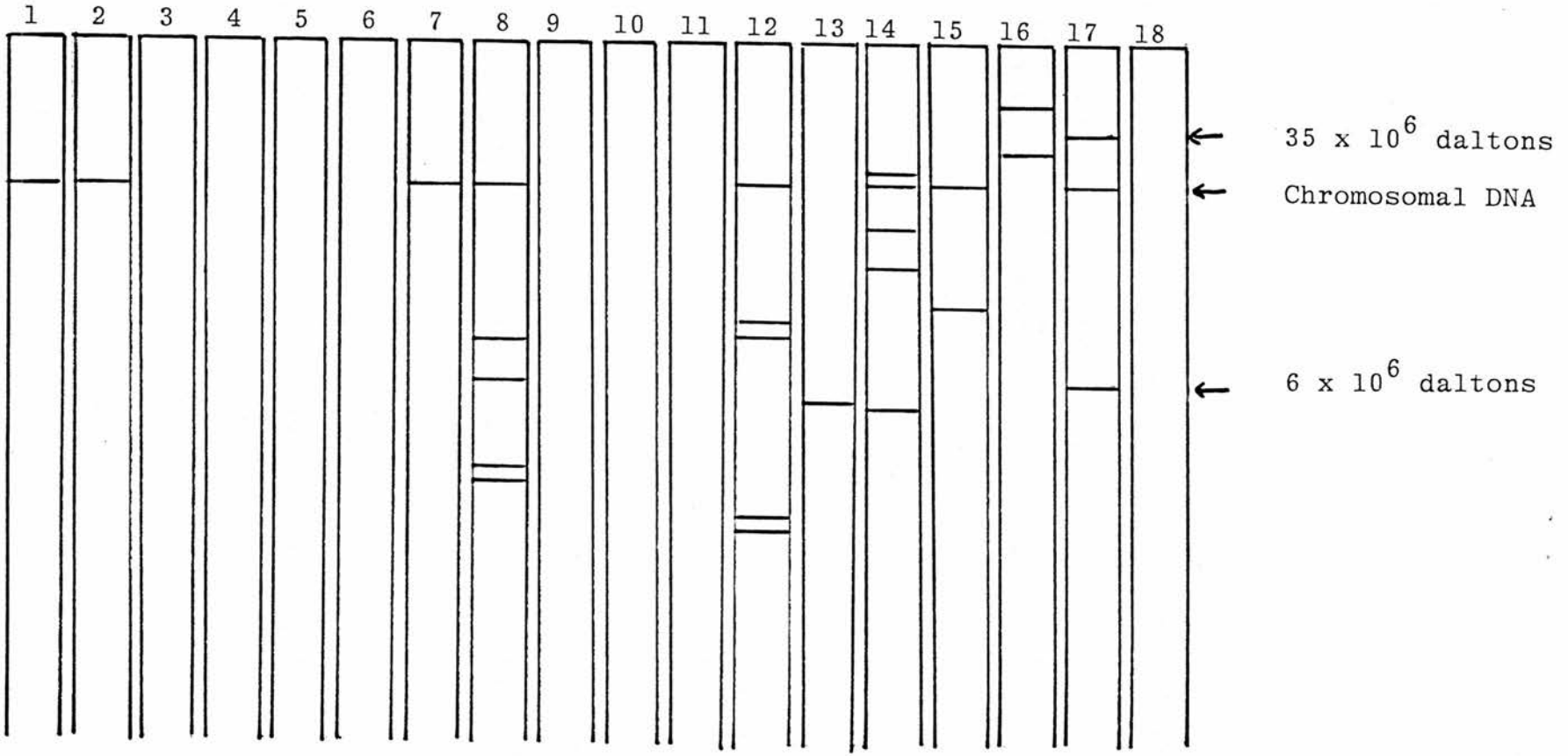


FIGURE 12. Schematic representation of Figure 11.

Table 7 lists the plasmid content of all the strains examined. With regard to the parental strains, 14 out of the 19 clinical isolates contained plasmids and of these 14, ten contained single plasmids. Strain K93 harboured 2 plasmids and strains K22, K76 and K97, 3 plasmids. Ten of the clinical strains contained plasmids which ranged in size from 2.5 to 35 Md. Analyses of the plasmid content of the 11 sewage strains revealed that 3 strains contained plasmids. Of these 3 strains SB38 contained 5 plasmids, the largest being 25.5Md the other 4 being less than 10Md. Strains SB68 and SB77 both cotained 4 plasmids ranging in size from 10.2 to 3.2Md.

Plasmid bands were cut out of low melting point agarose and digested seperately with the restriction enzymes Bam HI and Eco RI, in order to ascertain whether putative plasmid bands on the agarose gel were forms of the same plasmid since closed circular, open circular and nicked plasmid DNA migrate at different rates through agarose. Both digested and undigested plasmid DNA were run on a 0.8%(w/v) agarose gel and in most cases, analysis of the plasmid profiles indicated that the bands observed for each strain were due to individual plasmids and not to different forms of the same plasmid.None of the strains appeared to have identical plasmids based on restriction fragment patterns. However, not all plasmids appear to be cut by these 2 enzymes since in 3 cases K22 (2.9Md plasmid), K37 (3.2Md plasmid) and K48 (3.2Md plasmid) both digest and undigested plasmid migrate at the same rate through agarose. In the cases of plasmids that were cut more than once either of these enzymes, as was the case for the

TABLE 7

Plasmid content of strains examined for plasmid DNA.

PARENTAL STRAIN DESIGNATION	APPROXIMATE PLASMID WEIGHT (Md)	TRANSCONJUGANT DESIGNATION	APPROXIMATE MOLECULAR WEIGHT OF PLASMIDS FOUND IN TRANSCONJUGANT (Md)
K4	-	LA1	-
K22	34, 7.2, 2.9	LA2	-
K37	3.2	LA3	34, 3.2
K42	34	LA4	-
K45	34	LA5	-
K47	-	LA6	-
K48	3.2	LA7	34
K52	33	LA8	36
K54	33	LA9	-
K57	-	LA10	-
K58	35	LA11	-
K59	-	LA12	-
K65	-	LA13	-
K76	16.5, 6.7, 4.1	LA14	-
K78	26.5	LA15	28
K89	28	LA16	ND
K91	28	LA17	ND
K93	8.6, 3	LA18	-
K97	28, 7.6, 2.7	LA19	-
SB38	25.5, 8.3, 7.3 3, 2.8	LA20	-

TABLE 7 cont.

Plasmid content of strains examined for plasmid DNA.

PARENTAL STRAIN DESIGNATION	APPROXIMATE PLASMID WEIGHT (Md)	TRANSCONJUGANT DESIGNATION	APPROXIMATE MOLECULAR WEIGHT OF PLASMIDS FOUND IN TRANSCONJUGANT (Md)
SB62	-	LA21	-
SB65	-	LA22	NT
SB68	10.2, 9.4, 3.3 3.2	LA23	NT
SB74	-	LA24	-
SB77	9.4, 8.4 4.2, 4.1	LA25	NT
SB79	-	LA26	NT
SB80	-	LA27	-
SB88	-	LA28	NT
SA13	-	LA29	NT
SA92	-	LA30	NT

ND = NOT DETERMINED

- = NO PLASMIDS

NT = NO TRANSCONJUGANTS

34 and 7.2Md plasmids of K22 and the single plasmids of K45, K52, K58, K78 and K91, the sum of the fragments generated was the same as the molecular weight of the plasmid.

3.6. Hybridization of chromosomal and plasmid DNA to nick translated tet L and tet M probes.

Chromosomal and plasmid DNA were isolated from parental and transconjugant (parental x JH2-2) strains and analysed on a agarose gel. Chromosomal DNA was shown to be of high molecular weight since it migrated behind uncut bacteriophage DNA (48.5kb). Chromosomal and plasmid DNA was digested with the enzyme Hinc II and both cut and uncut DNA and controls were run on a 0.8%(w/v) agarose gel and the DNA subsequently transferred onto nitrocelluose paper. Hybridization of the transferred DNA to the nick translated tetracycline resistance probes tet L and tet M revealed no regions of sequence homology between the probes and the chromosomal and plasmid DNA isolated from the Group D streptococci. No hybridization was seen to the negative controls, pMV120 (tet N) and chromosomal DNA from JH2-2 (tetracycline sensitive). Hybridization was however seen between the positive controls, Hinc II cut and uncut plasmid pVB.A15 and pJI3 (containing tet L and tet M respectively) and the nick translated probes (Figures 13, 14).

3.7. Bacteriocin production

The presence of large conjugative plasmids in single antibiotic resistant strains of Group D streptococci could be indicative of the presence of bacteriocin production(Oliver,D et

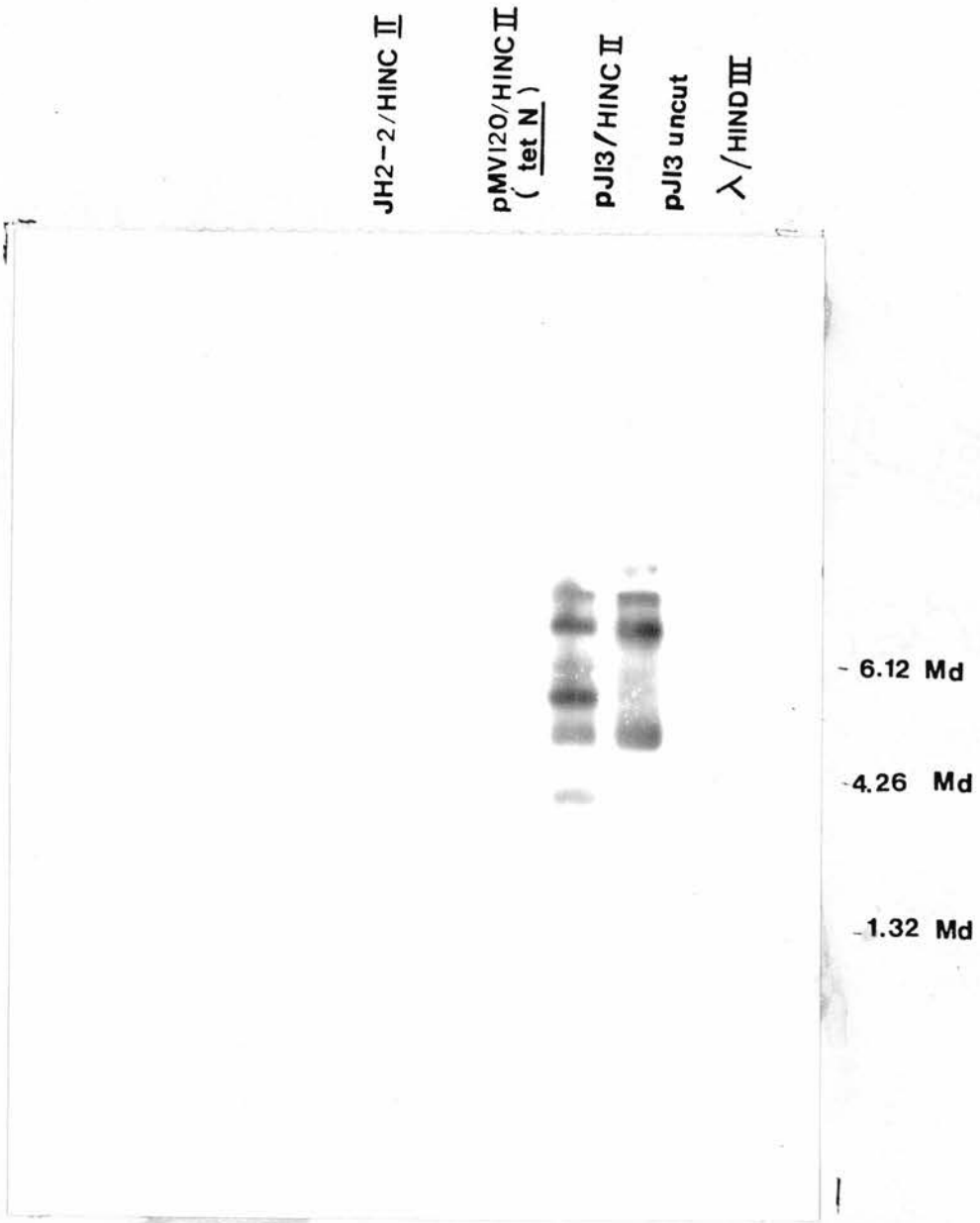


Figure 13. Hybridization of α-^{32}P nick translated tetM probe to chromosomal and plasmid DNA isolated from Group D streptococci.

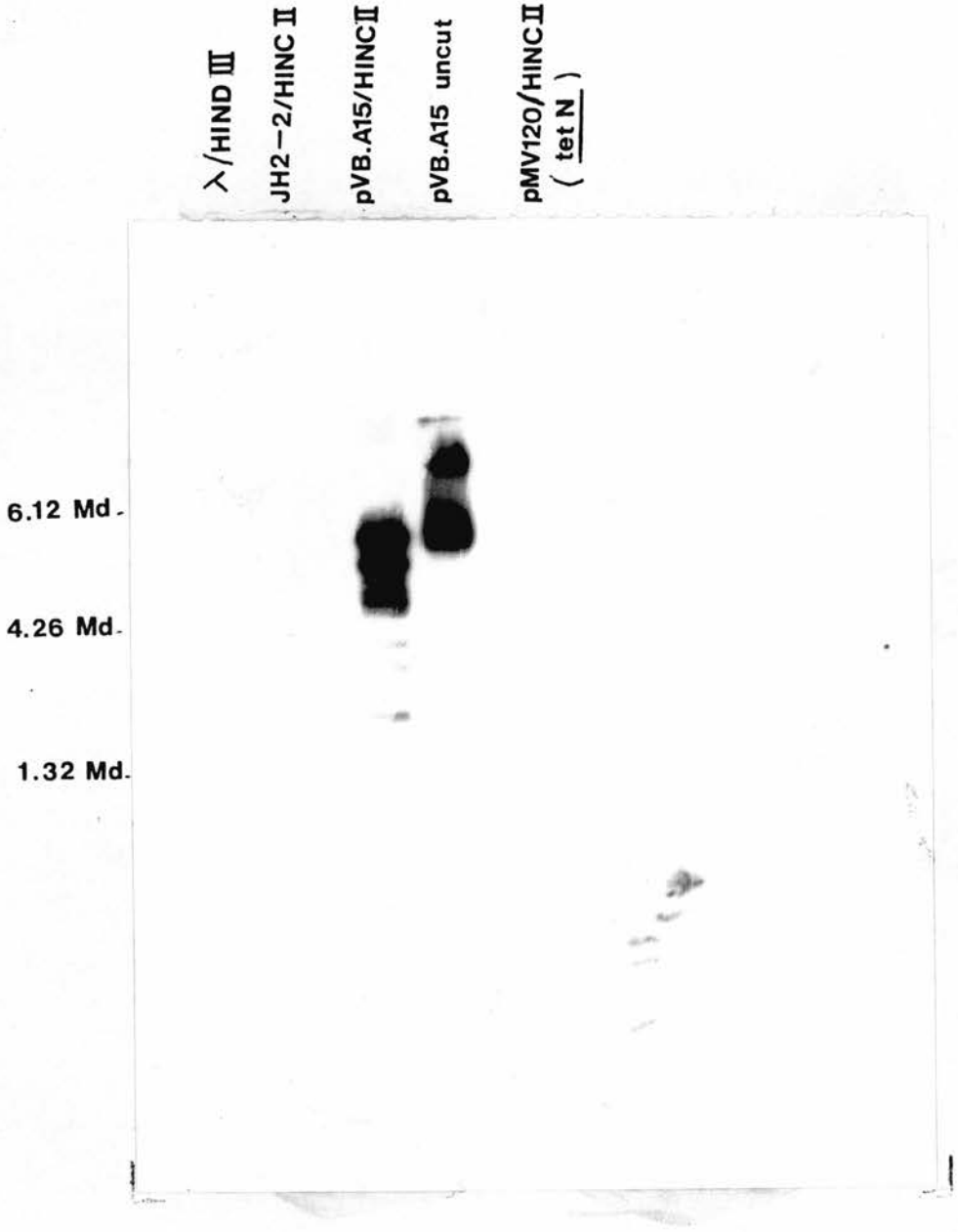


Figure 14. Hybridization of α-^{32}P nick translated tet L probe to chromosomal and plasmid DNA isolated from Group D streptococci.

al.,1977a) thus, parental and transconjugant strains were tested for this trait.The development of zone of inhibition of growth by the indicator
strain JH2 around the test strain was indicative of bacteriocin production. The only zone of inhibition was seen around the control strain containing the hemolysin-bacteriocin plasmid pJH2. None of the parental or transconjugant strains exhibited bacteriocin production.

3.8. Curing with Novobiocin.

The plasmid content of each of the 10 strains examined is shown in Table 8. None of the 10 strains studied showed curing of the tetracycline phenotype.However, all of the strains showed resistance to tetracycline. No spontaneous curing
of tetracycline resistance was observed in the untreated controls. Ten colonies from each strain that had undergone novobiocin curing were picked for plasmid analysis.Analysis of these colonies for plasmid DNA content was undertaken because if the plasmid DNA had been lost yet the colonies still exhibited a tetracycline resistant phenotype,then this would show that the resistance was carried on the chromosome.However,the plasmid profiles of the colonies picked were the same as that observed before treatment.In the case of the positive control JH-2,97 out of the 500 randomly picked novobiocin treated colonies showed tetracycline sensitivity indicating a curing frequency of 19.4%.Ten of the cured JH1-2 isolates were analysed for their plasmid content and all were found to have lost their 50Md plasmid which encodes tetracycline resistance. All of the strains

TABLE 8

Minimum inhibitory concentration of test strains to Novobiocin and their plasmid content.

STRAIN	MINIMUM INHIBITORY CONCENTRATION M.I.C. ($\mu g\ ml^{-1}$) OF NOVOBIOCIN	PLASMID CONTENT APPROXIMATE MOLECULAR WEIGHTS (Md)
K37	16	3.2
K42	32	34
K45	16	34
K48	16	3.2
K52	16	33
K54	16	33
K58	32	35
K78	16	26.5
K89	32	28
K91	16	28

maintained the same plasmid profile as was observed before curing.

4. DISCUSSION

4. DISCUSSION

This genetic survey of tetracycline resistance determinants in Fife, Scotland, examined both clinical and sewage isolates of Group D streptococci. No other surveys have been carried out on this group although a similar survey has been done on Group B streptococci (Burdett, V. 1980) and oral streptococci (Hawley, R.J. et al., 1980).

In this study the frequencies of transfer of tetracycline resistance in the absence of a sex factor were comaparable to the results obtained by other research workers (10^{-9} to 10^{-5} per donor) using Group D (Franke, A., & Clewell, D. 1981) and Group B (Burdett, V. 1980) streptococci. Plasmid DNA was demonstrated in 17 out of 30 parental strains and in 4 out of 23 tetracycline resistant transconjugant strains in this study. The plasmids ranged in size from 2.7 Md to 36 Md and the size of plasmids found in the parental strains often differed from those found in the corresponding transconjugant strains. This size difference could however be due recombination events.

No correlation was seen between plasmid size and transferability. This was of interest because many large plasmids i.e. with a size greater than 16 Md (Clewell, D.B., 1981a) found in Streptococcus faecalis are conjugative. However, large non-conjugative plasmids have also been reported (Oliver, D. et al., 1977a)

Individual plasmid bands were excised from low melting point agarose and digested with Eco RI and Bam HI separately. When these plasmids were analysed on agarose by gel electrophoresis, there was no change in their apparent molecular weights, suggesting that each plasmid band represented a single plasmid and not just a different form of the same plasmid. However, 3 of the plasmids did not appear to change their migration patterns when cut with either Bam HI or Eco RI, i.e. both linear and closed circular DNA migrated at the same rates through the agarose when electrophoresed. This would seem to suggest that these plasmids did not contain Bam HI or Eco RI recognition sites. Digestion with another restriction enzyme such as Hind III would show whether or not the plasmids lacking Bam HI and Eco RI recognition sites were indeed individual plasmids.

Nine parental strains showed a frequency of transfer of tetracycline resistance between 10^{-8} to 10^{-9} tranconjugant per donor. These strains were then subjected to a secondary transfer mating using the plasmid-less JH2-18 strain as the recipient, to see if the primary transconjugants could transfer their tetracycline resistance. Only one strain, strain LA15, contained plasmid DNA. In all nine cases the viable count for the tetracycline resistant donor strains was between 10^{-8} to 10^{-10} donors ml^{-1}.

These results were on a par with those previously observed with parental JH2-2 mating and they would seem to suggest that the tetracycline resistance transconjugants produced in both primary and secondary matings were not due to spontaneous

mutation to tetracycline resistance, but represented transfer of tetracycline resistance from a donor to recipient strain of Streptococcus faecalis.

Introduction of the sex factor pAMβ1 (carrying resistance to erythromycin) into the donor strains resulted in the mobilization of tetracycline resistance genes in 9 out of the 30 parental strains. Six of these strains were sewage isolates and they were unable to transfer their tetracycline resistance in the absence of the plasmid pAMβ1. There could have been several reasons why the determinant had not previously been transferred. It is possible that these six strains did not contain an origin of transfer sequence or they may have been unable to synthesize systems for DNA transfer, or replication. Of these 9 strains mobilized, only 3 contained plasmid DNA. The transconjugants containing pAMβ1 were not however screened for plasmid DNA content. No correlation was observed between either plasmid content and transferability of tetracycline resistance, in the presence of pAMβ1 or with plasmid size and tranferability of tetracycline resistance in the presence of pAMβ1. The plasmid pAMβ1 was selected for mobilization studies because in Burdett's study of Group B streptococci, this conjugative plasmid proved to be highly efficient in mobilizing the tetracycline resistance determinant. In her study, Burdett used several sex factors and she demonstrated that the frequency of transfer of plasmids carrying tetracycline resistance increased 100 to 10,000-fold when various sex factors were introduced into the donor strains. Franke and Clewell (Franke, A. and Clewell, D. 1981) observed a 100-fold increase in the frequency of tetracycline resistance

transfer in Streptococcus faecalis (Group D), when the sex factor pAD1 was present in the donor strain. In this case the genetic element encoding the resistance was not a plasmid but a chromosomal-borne transposon, Tn916. It was shown that Tn916 is able to exhibit conjugal transfer without the presence of a conjugative plasmid. The results of this thesis show a similar phenomenon in a number of strains, namely plasmid-less transfer of tetracycline resistance in both the absence and presence of the conjugative plasmid pAMβ1.

It is of interest to note that the introduction of pAMβ1 into 4 of the donor strains prevented the transfer of tetracycline resistance. This could have resulted because pAMβ1 may contain a regulatory protein which prevents transcription of the tetracycline resistance function. Alternatively, restriction of the tetracycline resistance determinant could take place.

A similar phenomenon has been reported by Clewell (Clewell, D. 1981a). He observed that the transmission of pAMβ1 was reduced when the plasmids pAMγ1 or pAD1 were also present in the donor strains. In the case of the author's four strains the erythromycin resistance was transferred but the tetracycline resistance was not.

It would be of interest to investigate the outcome of the introduction of other sex factors into donor strains to see if these sex factors could mobilise the tetracycline resistance

determinants.

Pheromone production was investigated since a negative result in the " clumping inducing" assay would confirm the low frequency of transfer of tetracycline resistance because pheromone production is only observed in the strains of Streptococcus faecalis with high frequencies of transfer i.e. 10^{-1} to 10^{-2} transconjugants per donor in broth matings, which contain conjugative plasmids (Clewell, D.B. 1981a). Thus, you would not expect to see self-clumping in strains exhibiting low frequencies of transfer and either lacking plasmid DNA or containing non-conjugative plasmids, which appeared to be the case in this study. Indeed, no clumping was seen when any of the parental strains were mixed with JH2-2 filtrates. Plasmid-less strains in fact acted as a negative control for experiments whilst Streptococcus faecalis strain DS5 provided a positive control showing clumping at a serial dilution of 1/32.

However,analysis of the results obtained from both the transfer studies and the plasmid content of the parental and transconjugant strains enables suggestions to be made as to thelocation of the tetracycline resistance determinants in a number of the parental stains studied;-

Strain K4- No plasmid DNA was evident in either the parental or transconjugant strain,LA1,and a moderate transfer frequency for the tetracycline resistance determinant was seen both in the presence and absence of the sex factor pAMβ1.The results suggest a chromosomal conjugative transposon aithough,the fact that the transfer frequencies were rather high,2.7×10^{-5} and 5.8×10^{-5} in the absence and the presence of the sex factor respectively,and

this is more indicative of a plasmid-borne determinant.Thus,it is possible that the determinant resides on a large plasmid present at a low copy number which is difficult to detect.

Strain K45- This strain exhibits a low frequency of transfer in the absence of pAMβ1 but a 100 fold increase in the frequency of transfer in the presence of the sex factor.Analysis of the plasmid content of the strain reveals the presence of a 34 Md plasmid in the parental strain but no plasmid DNA in the transconjugant LA5.This would seem to suggest that there are two determinants.A chromosomal one indicated by the low frequency transfer as seen in Tn916(Franke,A. & Clewell,D.B.,1981) and a second determinant on a non-conjugative plasmid capable of mobilisation by pAMβ1.However,the increase in the frequency of transfer seen with pAMβ1 could result from the transposition of the determinant from the chromosome to pAMβ1 followed by subsequent plasmid transfer.This hypothesis could be explored by investigating whether the tetracycline resistances are linked when re-tranferred from transconjugants.

Strains K58 and K93- Plasmid DNA was detected in the parental strains.A single 38 Md plasmid in the case of K58 and two plasmids of 8.6 and 3 Md in the case of K93.No plasmid DNA was found in their transconjugants.Both strains displayed a low frequency of transfer in the absence of pAMβ1 and no tranfer in the presence of the sex factor.These results indicate that the tetracycline resistance determinant resides in the chromosome and is capable of conjugative transposition.However,the presence of pAMβ1 results in the inhibition of the transfer of the tetracycline resistance determinant.These conclusions can be extended to cover strains SB80 and SB74,two strains which contain

no plasmid DNA but which display similar frequencies of tranfer to strains K58 and K93, in the absence of pAMβ1.

Strains K78 and K52- A low frequency of transfer was observed both in the presence and absence of pAM 1 and plasmid DNA was present in both the parental and transconjugant strains. This would seem to suggest that the resistance was plasmid-borne in these two strains. This conclusion may also be applied to strains K37 and K48 since it is possible that these two strains may carry large low copy number plasmids which were unable to be detected in these studies.

Strains K59 and K65- In the case of these two strains a low frequency of transfer was observed in the absence of pAMβ 1. However a 100 fold increase in the frequency of transfer was seen in the presence of pAMβ1. No plasmid DNA was observed in either the parental or the transconjugant strain. A chromosomal-borne tetracycline resistance determinant is suggested by these results. This chromosomal resistance is capable of conjugative transposition. The apparent increase in the frequency of transfer in the presence of pAMβ1 would seem to indicate that the tetracycline resistance determinant is also capable of transposition to pAMβ1.

Strain SA13- No transfer of tetracycline resistance was seen either in the presence or absence of pAMβ1 and no plasmid DNA was detected. These results suggest that the tetracycline resistance determinant is a non-transferable chromosomal-borne determinant.

As mentioned previously (1.2.5), a number of tetracycline resistance determinants have been identified and two, tet M (non-plasmid associated) and tet L (associated with small non-conjugative plasmids) have been cloned into Escherichia

coli plasmids for use as genetic probes (Burdett, V. et al., 1982a,b). The cloning of the tetracycline resistance genes from streptococci into Escherichia coli plasmids reduces the cross hybridization of common sequences found in streptococci allowing specific hybridization to homologous sequences.Using the tet M and tet L probes it has been shown that the tet M-like determinants are widespread in nature(Burdett,V.et al.,1982b).

In this study, chromosomal and plasmid DNA was isolated from parental and transconjugant strains and 5 µg and 100 µg respectively of each DNA was cut with Hinc II before electrophoresing both cut and uncut DNA through 0.8%(w/v) agarose. The enzyme Hinc II was used because Burdett has shown that the tetracycline resistance determinants tet L and tet M are contained within Hinc II fragmenta (Burdett, V. et al., 1982a).

In order to maximize the detection of hybridization of nick translated probes to the chromosomal DNA, the amount of chromosomal DNA (5 µg) loaded onto the agarose gel was made equivalent to that of the plasmid DNA (100 ng) because the molecular weight of the streptococcal chromosomal DNA is approximately 1,000 times greater than that of the average plasmid detected in this study. Therefore, the probability of detecting a gene sequence on the chromosomal DNA is less than that for its detection on plasmid DNA because of the difference in the size of the genome.

In an attempt to try and distinguish the presence of large low copy number plasmids carrying tetracycline resistance from

chromosomally encoded tetracycline resistance, digested chromosomal DNA was run alongside undigested DNA, because in theory large supercoiled plasmids should run ahead of chromosomal DNA after electrophoresis. Thus, hybridization to uncut DNA should reveal whether or not the nick translated probes hybridize to plasmid of chromosomal DNA. In the case of chromosomal DNA hybridization, the band of hybridization seen after digestion with Hinc II should run faster than that observed with uncut chromosomal DNA. However, it should be remembered that high molecular weight plasmids present in low copy numbers are frequently nicked during isolation and therefore run with chromosomal DNA.

Hybridization studies did not reveal homology between the cloned tet L and tet M determinants and the strains of group D streptococci under investigation. The hybridization conditions used in this study appeared to be suitable since hybridization was seen with pVBA.15 and pJI3 carrying the tet L and tet M determinants respectively.However,it is possible that the hybridizing conditions and the nitrocellulose filter washing conditions were too stringent for the probes since the conditions would only detect sequences with 80 to 100% sequence match.Thus,if the probe was less than 80% homologous to the DNA bound to the nitrocellulose filter paper then hybridization would not have been detected.It was hoped that the hybridization studies would show that the tetracycline resistance determinants from the U.S.A.showed homology to those from Fife,Scotland,indicating a common ancesteral origin and with this in mind,it is of intrest to note that in another study where the

broad geographical distribution of hemolysin-bacteriocin plasmid DNA was investigated(LeBlanc,D.J. et al.,1983) a strain from London,England,JH1, harbouring the plasmid pJH2 (Jacob,A.et al.,1975)and the strain DS16 from Michigan,U.S.A.(Tomich,P.K. et al.,1979)harbouring pAD1 were shown to share at least 90% homology with each other.These two strains also carry a chromosomal-borne tetracycline resistance determinant and this tetracycline resistance determinant shows some degree of homology between the two strains,indicating that the determinants share a common ancesteral origin.However,the degree of homology between the determinants was not indicated by the authors(LeBlanc,D.J. et al.,1983).

It is also possible that the tetracycline resistance determinants examined in this study show homology to other reported gram-positive tetracycline resistant determinants such as tet N which was shown to reside on the large conjuagtive plasmid pMV120 isolated from Streptococcus agalactiae (Burdett, V. et al., 1982) or to the chromosomal tetracycline resistance determinants found in Streptococcus faecalis JH1 (Le Blanc, D.J. & Lee, L.N. 1982). However, at present the homology between other reported tetracycline resistance determinants in gram-positve bacteria and those identified by this study cannot be investigated because to date, only tet L and tet M have been cloned (Burdett, V. Personal communication). It has been suggested that tetracycline resistance transfer in a number of strains in this study may be transposon mediated but the results of the hybridization studies would seem to indicate that these resistance determinants share no homology, under the stringency conditions used in the study, with the tetracycline resistance

transposon Tn916 which contains the tet M determinant (Burdett, V. et al., 1982a,b) and as a consequence the determinants in this study must differ from the tetracycline resistance transposons Tn918 and Tn919, which share extensive homology to Tn916 (Clewell, D.B. et al., 1985; Fitzgerald, G.F. & Clewell, D.B. 1985).

Bacteriocin production was investigated because if the plasmids did not code for tetracycline resistance, there was the possibility that they could code for bacteriocin production. bacteriocin production and hemolysin production are often associated together on the same plasmid, although Oliver (Oliver, D. et al 1977a) has reported the existence of a 28 Md non-conjugative plasmid encoding solely bacteriocin production. Since bacteriocin production in streptococci is normally encoded by large plasmids this could provide a possible function for the large plasmids in this study if indeed they were not involved in the generation of tetracycline resistance. However, none of the parental or transconjugant strains investigated demonstrated bacteriocin production.

Curing of tetracycline resistance using the antibiotic novobiocin was performed on all ten isolates containing a single plasmid in an attempt to ascertain whether or not the tetracycline resistance was encoded on a plasmid. Novobiocin has been shown to inhibit in vitro the activity of DNA gyrase isolated from Escherichia coli (Gellert, M., et al., 1976). The enzyme DNA gyrase introduces negative superhelical turns into double-stranded relaxed closed circular DNA, thus novobiocin

provides a plasmid site specific curing againt. This curing method was selected over others because of its reported high efficiency of curing plasmid DNA in Streptococcus faecalis strains of 10 to 34% (McHugh, G. & Swartz, M., 1977) as compared with 0.14% by incubation at 45°C, 0.07% using acriflavine, 0.04% with ethidium bromide and 0.16% with ultraviolet light (Jacob, A. & Hobbs, S., 1974). However, in this study none of the 10 strains showed any degree of curing. A random sample of 10 colonies grown from novobiocin treated strains were screened for plasmid content.These ten strains containing a single plasmid were selected because if curing of tetracycline resistance was achieved concomitant with the loss of plasmid DNA this would indicate that the determinant resided on the plasmid.However,none of the novobiocin treated strains had lost their single plasmid DNA, but it is possible that the selected colonies were not representative of the true frequency of curing. In theory at least 100 colonies should have been screened for plasmid DNA in order to obtain a valid frequency of curing. Alternatively there is the possibility that lysis of the bacterial cell wall was incomplete during plasmid isolation therefore the plasmids went undetected, even thought the plasmid containing control strain, Streptococcus faecalis DS5, showed all three of its plasmids, thus indicating successful cell wall lysis.

Clewell and co-workers (Clewell, D.B. et al 1974) demonstrated that Streptococcus faecalis strain DS5 containing plasmids pAMα1 (encoding tetracycline resistance), pAMγ1 (encoding hemolysin and bacteriocin production) and pAMβ1 (encoding erythromycin resistance) could only be cured of plasmid

pAMβ1 (present in 1 or 2 copies per chromosome genome) using acridine orange or ethidium bromide. Clewell suggested that this could be due to the relatively high copy numbers of plasmid pAMα1 (9 copies) and pAMγ1 (5 copies) per chromosome genome equivalent, and perhaps the presence of multiple copies of a plasmid may help to give some guarantee against loss by segregation. In general small plasmids are present at higher copy numbers than the large plasmids. In this study, two of the 10 strains under investigation contained low molecular weight plasmids, so it may be worth looking at their copy numbers using the method employed by Clewell, as this could affect the curing efficieny.

The results of the experiments described in this thesis have not demonstrated the exact location or nature of the tetracycline resistance determinants in the Group D streptococci investigated.However,analysis of the results of the transfer frequencies and sex factor studies have enabled suggestions to be made as to the location of the tetracycline resistance determinants in a number of the strains.The results have shown a number of other things that reinforce observations made by other investigators, such as the lack of pheromone production in cases where the frequency of transfer of tetracycline resistance is low and in the absence of conjugative plasmids(Clewell,D.,1981a). The results have also demonstrated that the tetracycline resistance determinants do not appear to share any sequence homology with two cloned streptococcal resistance determinants tet L and tet M at least at the stringency conditions used in this study.

There are a number of ways to further investigate the

location and nature of these determinants and so extend these preliminary studies. Cloning of the determinants present in the strains under investigation would enable a more detailed analysis of the nature of these determinants using the methods described by Burdett and colleagues (Burdett, V. et al., 1982a,b) which involves deletion analysis and cloning of the determinant into an Escherichia coli vector to prevent cross hybridization with other streptococcal sequences. Obviously, only 1 or 2 tetracycline resistance determinants would be cloned i.e. 1 from a strain containing a single known tetracycline resistance encoding plasmids and 1 from a plasmid-less strain showing tetracycline resistance. Before attempting to clone the plasmid encoded determinant it would of course be necessary to confirm that indeed tetracycline resistance was plasmid-borne in that particular case therefore transformation experiments should be performed using the Streptococcus sanguis Challis strain (Le Blanc, D. & Hassell, F.P. 1976). However, if no tetracycline resistant transformants are obtained this does not mean that the tetracycline resistance determinant is not plasmid-borne. When Le Blanc and Hassell (Le Blane, D. and Hassell, F.P. 1976) attempted transformation of the Streptococcus sanguis Challis strain with plasmids p AMβ1 (encoding erythromycin resistance) p AMα 1(encoding tetracycline resistnace) and pAMγ1 (encoding hemolysin production) derived from Streptococcus faecalis strain DS5 (Clewell, D. et al., 1974) only the pAMβ1 plasmid was found to transform the Challis strain. Le Blanc and Hassell suggested that the tetracycline resistance phenotype encoded by pAMα1 may lack the ability to be expressed in the Challis strain. Alternatively, the plasmid may require a host replicative function provided by

Streptococcus faecalis that is absent in Streptococcus sanguis. Thus, there is no guarentee that transformation of tetracycline resistance plasmids into the Challis strain would result in the expression of the tetracycline resistance determinant encoded on the plasmids in this study.

It should however by borne in mind that if the transformation experiments establish that the plasmids present in the strains under investigation do indeed code for tetracycline resistance, extensive curing procedures should be untaken since if the strains are still resistant to the antibiotic, this may indicate that the strain harbours a second tetracycline resistance gene and this might interfere with cloning of the tetracycline resistance determinant. A strain carrying two tetracycline resistance determinants has recently been described by Le Blanc and Lee (Le Blanc, D. & Lee, L. 1982). They demonstrated that Streptococcus faecalis strain JH1 carries two tetracycline resistance determinants. One is carried on a plasmid and mediates a constitutive resistance to the antibiotic. The second determinant, present in a plasmid-cured strain of JH1, mediates an inducible tetracycline resistance and is located either on the chromosome or on an undetectable plasmid. The possibility that tetracycline resistance may be carried on a undetectable plasmid should also be considered from the results obtained in this study. The plasmid may be undetectable because it is present in a low copy number in the parental strain, and it may be lost during the plasmid isolation procedure. Alternatively, the plasmid may become nicked during isolation and as a consequence it may run with chromosomal DNA during gel

electrophoresis.

An investigation of inducibility of tetracycline resistance amongst the group D strains in this survey would be of value, because with the exception of Le Blanc and Lee, all the reports of tetracycline resistance in streptococci have been of a constitutive nature. Recently Burdett has shown differences in the accumulation of tetracycline between different tetracycline resistance determinants (Burdett, V. et al., 1985). Tet L containing cells do not accumulate tetracycline, whereas cells containing tet M or tet N do. This would seem to suggest a variation in the mechanism of tetracycline resistance between different determinants. In the case of Escherichia coli (Levy, S.B. 1981) it has been shown that although the tetracycline resistance determinants may differ genetically, their mechanism of tetracycline resistance is the same.

If the tetracycline resistance genes in the apparently plasmid free strains in this study are chromosome-borne, then it should be possible to show whether or not the resistance is encoded by transposon using the methods described by Gawron-Burke and Clewell(Gawron-Burke,C.& Clewell,D.B.,1982).

Another possibility worthy of examination is that tetracycline resistant plasmid-free strains could harbour a plasmid capable of reversible integration into the host chromosome, since in this study 17 tetracycline resistant donor strains contained plasmid DNA yet only 4 of their transconjugants were shown to harbour plasmids. The other plasmid-free donor and

transconjugant strains could contain an integrated plasmid carrying the tetracycline resistance genes.

This phenomenon has been well documented in the case of the Hfr strain Escherichia coli K12 (Glass, R.E., 1982) in which the 63 Md F factor becomes integrated into the host chromosome. This inegration is reversible and when the F factor is excised from the chromosome it often takes with it DNA sequences derived from the host chromosome and is known as an F' factor. This F' plasmid is capable of autonomous replication the same as normal plasmids. Obviously, the addition of host DNA to the F plasmid in F' formation causes a change in the molecular weight of the plasmid. Chromosomally integrated plasmids have also been reported in Haemophilus pneumoniae (Roberts, M.C. & Smith A.L. 1980; Stuy, J.H., 1980).

If the tetracycline resistance plasmids in the strains in this survey have integrated into the chromosome then probing the chromosome of plasmid-free strains using a α-^{32}P labelled plasmid probe constructed from the relevant tetracycline resistant determinant should demonstrate chromosomal integration.

This discussion so far has assumed that plasmids carried by the strains in this study code for tetracycline resistance. Some strains contain a number of different plasmids presumably with different phenotypes. If these plasmids do not encode tetracycline resistance then what do their genes code for? They do not code for bacteriocin-production or pheromone responses. They could however code for resistance to another

antibiotic although these strains have been shown to be sensitive to gentamicin, ampicillin, penicillin, streptomycin, and erythromycin. All the parental strains are also sensitive to chloramphenicol except for strain K76. In this survey 15 out of 38 clinical isolates and their transconjugants carried a plasmid with a molecular weight of between 25.5 to 36 Md, yet in only 3 cases was this plasmid transferred to the transconjugant. Out of the 11 sewage isolates only one strain carried a large plasmid of 25.5 Md and this was not transferred.

Apart from indicating the existance of a transposon encoded tetracycline resistance, analysis of the transconjugants containing sex factor pAMβ1 using gel electrophoresis could reveal that plasmids from the donor strains had been mobilized into the transconjugants. It should be remembered that the frequency of transfer of tetracycline resistance was used as the criterium to judge whether mobilization had occurred and if the plasmids present in the donor strains encode another phenotype then this would not have been detected.

If the smaller plasmids in the strains do not code for tetracycline resistance then are they cryptic? The smallest plasmid demonstrated had a molecular weight off 2.7 Md. This is sufficient to code fo 2 to 3 proteins. Many small and large cryptic plasmids have been reported in Group D streptococci.

The possibility of transfer of tetracycline resistance by transduction or transformation could be investigated using the regimes described by Jacob and Hobbs (Jacob, A. and Hobbs, S. J.

1974) although these two types of gene transfer have not been reported in Group D streptococci.

5. CONCLUSION

5. CONCLUSION

In conclusion, the results of this survey indicate a plasmid-less transfer of tetracycline resistance in a number of strains possibly via a conjugal transposon like Tn 916 (Franke, A. & Clewell, D. 1981) which has been demonstrated in Streptococcus faecalis strain DS16. There have been may reports of plasmid-less tetracycline resistance transfer in the Genus Streptococcus. However, tetracycline resistance could be carried on a plasmid capable of reversible integration into the host chromosome or even carried on a large undetectable plasmid present at a low copy number.Although the location of the tetracycline resistance determinant has been suggested in a number of strains,further experiments need to be performed to precisely locate and characterise the tetracycline resistance determinants carried by the strains in this study.

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